22nd International Conference on Oxygen Binding and Sensing Proteins

(O2BiP)

University of Essex, Wivenhoe Park, Colchester, Essex, United Kingdom

26th – 29th August 2025

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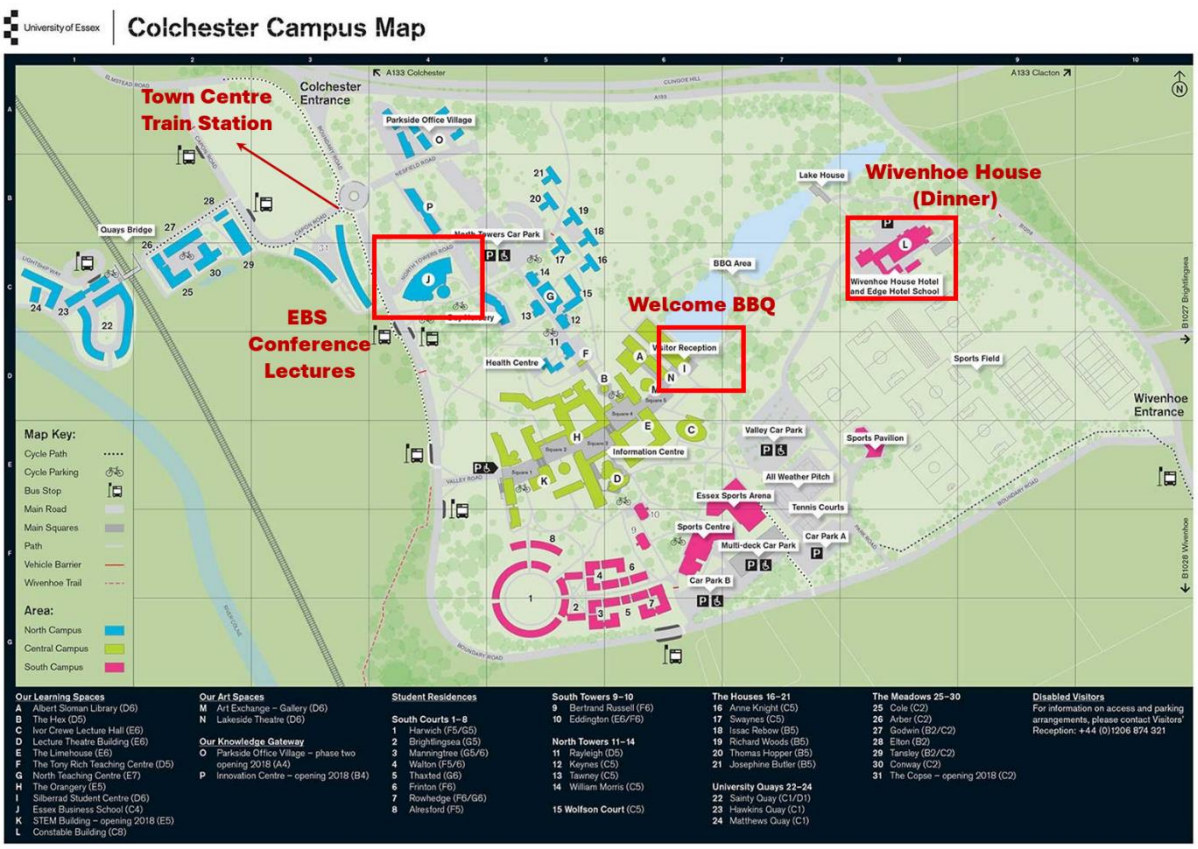

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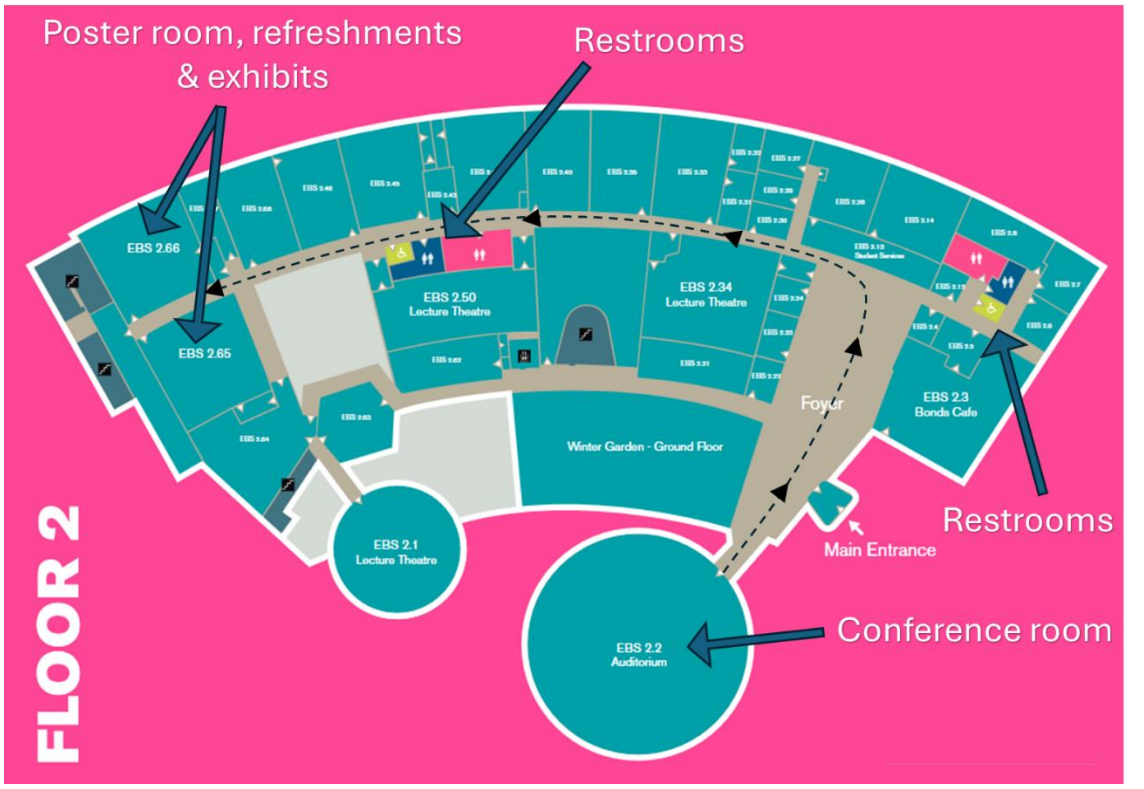
Conference, Posters and Exhibitor Rooms

EBS (Essex Business School) Rooms 2.2 (Auditorium)

2.65 and 2.66 (Posters, Refreshments and Exhibitors)



Social Events and Practical Workshop

Tuesday 26th Evening BBQ: Silberrad Centre

Please join us for food and drink at the Siberrad Centre next to the lakes of Wivenhoe Park. Wivenhoe Park is a striking landscape of more than 220 acres. Wivenhoe Park is home to a range of plants and wildlife including some extraordinary and historic trees. Our three artificial lakes provide homes for carp, cormorants, and kingfishers.

World-famous painter John Constable immortalised Wivenhoe Park in his 1816 painting. Much of the landscape is older still, from medieval oaks to the grand Wivenhoe House of 1758.

Wednesday 27th Evening: Excursion to Colchester Castle

Colchester Castle is a medieval Norman fortress in Colchester, Essex, dating back to the late 11th century. Its remarkably well-preserved keep is the largest of its kind in Europe, built atop the foundations of the Roman Temple of Claudius from the 1st century CE.

The castle has witnessed pivotal moments in English history. In 1216, it withstood a 3-month siege by King John after his breach of the Magna Carta. During the English Civil War in 1648, it played a central role in the Siege of Colchester. Royalist forces were besieged for 11 weeks by Parliamentarians. After the Royalists surrendered, commanders Sir Charles Lucas and Sir George Lisle were executed on castle grounds.

Over the centuries, Colchester Castle has served as a prison and, later, a garden pavilion. It has housed Colchester Museum since 1860.

Feel free to stay and eat at one of Colchester's many City Centre restaurants just a few minutes walk away.

Thursday 28th Conference Social Diner: Wivenhoe House

We hope you will join us for the conference dinner at Wivenhoe House, a Georgian manor set within the picturesque grounds of the University of Essex. Built in 1759 and famously painted by John Constable, the house blends historic charm with modern hospitality. Over the years, it has welcomed distinguished guests including Winston Churchill and Queen Elizabeth II.

During World War II, Wivenhoe House served as a military headquarters, including for the 2nd SAS Regiment during preparations for D-Day. In 1964, the estate became part of the University of Essex and was later transformed into a four-star hotel and the home of the Edge Hotel School, where students gain invaluable hands-on experience in hospitality management.

Friday 29th Practical Workshop

Biophysical Methods for Studying Oxygen-Binding Heme Proteins

Designed for early career scientists including PhD students and Postdoctorates, the afternoon workshop will provide hands-on focused training in advanced techniques for investigating oxygen-binding heme proteins. Participants will gain practical experience in:

- **Oxygen Consumption in Turbid Samples (Mark Shepherd)**

Learn to measure oxygen binding and release in challenging systems like whole cells or membrane preparations using optical and electrochemical methods.

- **Rapid Reaction Kinetic Spectroscopy (Brandon Reeder)**

Master rapid kinetic techniques to analyse ligand binding and rapid reaction kinetics in the sub-second timescale.

- **EPR Spectroscopy of Metalloproteins (Dima Svistunenko)**

Explore electronic and spin states of metal centres, with practical training in EPR and spectral interpretation.

A sign-up sheet will be at the registration desk and poster room. Space is limited to ~15 people so will be on a first come-first served basis.

22nd International Conference on Oxygen Binding and Sensing Proteins (O2BiP) Conference Program (EBS 2.2)

Tue 26 - Fri 29 Aug 25

Tuesday 26th August 2025

15:00-17:00 Registration

17:00-19:00 Welcome BBQ, Silberrad Centre Plaza

Wednesday 27th August 2025, Rooms: EBS 2.2, 2.65 & 2.66

08.30-17.00 Registration

08:40 - 09:10 Tea/Coffee, EBS 2.65 & 2.66

09:10 -09:20 *Welcome - Brandon Reeder, School of Life Sciences, University of Essex*

09.20-13.00 Session 1: Bacterial heme proteins and heme based sensors, EBS2.2

Chairpersons: Angelo Fago, Dimitri Svistunenko

09:20-09:45 *Anaerobic and aerobic sulfide binding to human neuroglobin: sulfheme derivatives*

Federico Sebastiani, Department of Chemistry, University of Florence, Florence, Italy

9:45-10:10 *Dissecting the conformational complexity of a prokaryotic heme transporter*

Schara Safarian, Max Planck Institute of Biophysics, Frankfurt am Main, Germany

10:10-10:35 *From antibacterial strategy to modulation of hemoglobin oxygen affinity: the unexpected dual role of C35*

Serena Faggiano, Department of Food and Drug, University of Parma, Parma, Italy

10:35-11:00 *Anaerobic heme catabolism in three bacterial pathogens*

Kirsten Wolthers, Department of Chemistry, The University of British Columbia, Canada

11.00-11.30 Morning Refreshments and Posters, EBS 2.65 & 2.66

11:30-11:55 *The high diversity of oxygen reactivity in bacterial terminal oxidases*

Petra Hellwig, Faculty of Chemistry, University of Strasbourg, Strasbourg, France

11:55-12:20 *Targeting aerobic respiratory chains to combat bacterial pathogens*

Mark Shepherd, School of Biosciences, University of Kent, Canterbury, United Kingdom

12:20-12:50 *Abstract selected speakers:*

12:20-12:35 *The bd-type Cyanide Insensitive Oxidase from the multidrug-resistant pathogen Pseudomonas aeruginosa: interaction with gaseous ligands*

Elena Forte, Department of Biochemical Sciences "A. Rossi Fanelli", Sapienza University of Rome, P. le A. Moro 5 00185 Rome, Italy

12:35-12:50 *Targeting cytochrome bd oxidases from Acinetobacter baumannii with steroid drugs.*

Guy Joiner, School of Biosciences, University of Kent, Canterbury, United Kingdom.

12.50-14.00 Lunch and Poster Session, EBS 2.65 & 2.66

14.00-17.20 Session 2: Evolution of Oxygen Binding Proteins, EBS2.2

Chairpersons: Michael Berenbrink

14:00-14:25 *Conformational analysis of human and crocodilian Hb by cryo-electron microscopy*

Jeremy Tame, Department of Biology and Biochemistry, Yokohama City University, Yokohama, Japan

14:25-14:50 *Ngb function in Zebrafish – lessons learned from a knockout model*

Andrej Fabrizius, Department of Biology, University of Hamburg, Germany

14:50-15:15 *Convergent reductions in hemoglobin buffering power in lineages of small, high-metabolic rate birds and mammals: implications for O_2 delivery*

Kevin Campbell, Department of Molecular Physiology and Biophysics, University of Iowa, Iowa, United States of America.

15:15-15:40 *Convergent reductions in hemoglobin buffering power in lineages of small, high-metabolic rate birds and mammals: implications for CO_2 elimination*

Michael Berenbrink, Institute of Infection, Veterinary & Ecological Sciences University of Liverpool, United Kingdom

15:40 - 16:10 Afternoon refreshments and Posters, EBS 2.65 & 2.66

16.10 -17.20 *Abstract selected speakers:*

16.10-16.25 *Characterization of bryophyte hemoglobins provides insight into ancestral functions*

Ryan Sturms, Drake University Department of Chemistry and Physics. Des Moines, USA

16.25-16.40 *The Role of Globin Proteins in the Transition from Water to Land*

Soraya Safavi, Institute of Cell and Systems Biology of Animals, University of Hamburg, D-20146 Hamburg, Germany

16.40-16.55 *Resurrecting lost ancient myoglobins to elucidate mechanisms of diving adaptation in mammals*.

Hiroshi Imamura, Department of Bio-Science, Nagahama Institute of Bio-Science and Technology, 1266 Tamura, Nagahama, Shiga 526-0829, Japan

18.00-19.30: **Evening excursion to Colchester Medieval Castle.**

Thursday 28th August 2025

09:00 - 09:20 Tea/Coffee, EBS 2.65 & 2.66

09:20-13:00 Session 3: Structure, Function, and Dynamics, EBS2.2

Chairpersons: Mark Shepherd, Luca Ronda

09:20-09:45 *Phytoglobins - some new insights into their structure and biological function*

Leif Bulow, Department of Chemistry, Lund University, Lund, Sweden

09:45-10:10 *Heme-based dioxygenases: Structure, function and dynamics*

Syun-Ru Yeh, Department of Biochemistry, Albert Einstein College of Medicine, New York, United States of America.

10:10-10:35 *Structural insights into the functional properties of cytochrome c oxidase*

Denis Rousseau, Department of Biochemistry, Albert Einstein College of Medicine, New York, United States of America.

10:35-11:00 *Cytochrome b5 reductase 4 efficiently reduces Neuroglobin and Cytoglobin*

Jesús Tejero Department of Medicine, University of Pittsburgh, Pittsburgh, USA

11:00-11:30 Morning Refreshments and Posters, EBS 2.65 & 2.66

11:30-11:55 *Candida albicans Utilises Methaemoglobin to Build Ultra-Drug Resistant Polymicrobial Biofilms*

Campbell Gourlay*,* School of Biosciences, University of Kent, Canterbury, United Kingdom

11:55-12:55 *Abstract selected speakers:*

11.55-12.10 The multiplicity of the Caenorhabditis elegans globin family.

Bart Braeckman, Biology Department, Ghent University, Belgium

12.10-12.25 *The enhanced hemoglobin function can be explained by additional tertiary structural changes exerted by changes in hydrophobicity in the α1β1 interface*.

Antonio Tsuneshige, Department of Frontier Bioscience and Research Center for Micro-Nano Technology, Hosei University, 3-11-15 Midori-cho, Koganei-shi, Tokyo 184-0003, Japan

12.25-12.40 Characterization of globin Y in vertebrates.

Cäcilia Plate, Institute of Cell and Systems Biology of Animals, University of Hamburg, D-20146 Hamburg, Germany.

12.40-12.55 Hemoglobin haem capture by staphylococcal receptor IsdB: a complex process unveiled by time-resolved X-ray solution scattering.

Marialaura Marchetti, Institute of Cell and Systems Biology of Animals, University of Hamburg, D-20146 Hamburg, Germany.

12:55-13:50 Lunch and Poster Session, EBS 2.65 & 2.66

13:50-17:20 Session 4: Heme-proteins In Health and Diseases, EBS2.2

Chairpersons: David Hoogewijs, Brandon Reeder

13:50-14:15 *Cytoglobin in Hepatic Stellate Cells Plays an Anti-fibrotic Role in Chronic Liver Injury*

Norifumi Kawada, Department of Homeostatic Regulation and Liver Cancer Treatment, Graduate School of Medicine, Osaka Metropolitan University, Osaka, Japan

14:15-14:40 *Targeting Nitric Oxide-Driven Cancer Progression: Hemin Derivatives Suppress Migration and Angiogenesis via Redox Pathway Modulation*

Amir Abdo, Research Ireland Centre for Medical Devices, University of Galway, Ireland.

14:40-15:05 *Polymerised bovine Hb used as a perfusion fluid for organ preservation*

Stefano Bruno, Department of Food and Drug Sciences, University of Parma, Parma, Italy

15:05-15:30 *Androglobin in basal metazoans: ancient functional association with cilia*

Carina Osterhof, Department of Endocrinology, Metabolism and Cardiovascular system, University of Fribourg, Fribourg, Switzerland.

15:30 - 16:00 Afternoon refreshments and Posters, EBS 2.65 & 2.66

16:00 -16:25 *Cytoglobin as a mediator of non-canonical redox signaling*

Jian Cui, Helmholtz-Munich, Germany

16:25 -16:50 *Cytoglobin controls cardiac morphogenesis by regulating NO-sGC signaling.*

Paola Corti, School of Medicine, University of Maryland, USA

16:50 -17:35 *Abstract selected speakers:*

16:50 -17:05 *Neuroglobin and Cytoglobin – Investigation of Functional role in the retina.* **Ranjan Rajendram.** University College, London, UK

17:05 -17:20 *Cytoglobin: A potential respiratory regulator in retinal cells.*

Alex Binderup Sort Jensen, Section for Zoophysiology, Department of Biology, Aarhus University

17:20 -17:35 *Heme Modulation of p53 and p63: Structural and Functional Insights.*

Artur Sergunin, *Faculty of Science, Charles University, Hlavova 2030/8, 128 00, Prague, Czech Republic*

19:30 – 21:30 Social Dinner, Wivenhoe House

Friday 29 August 2025

09:00 - 09:20 Tea/Coffee, EBS 2.65 & 2.66

09:20-13:00 Session 5: Emerging topics in oxygen binding/sensing proteins, EBS2.2

Chairpersons: Stefano Bruno

09:20-09:45 *Illuminating Heme Loss from Hemoglobin: New Fluorescent Tools to Track Heme-Iron Flow During Infection*
Robert Clubb, Department of Chemistry and Biochemistry, University of California, USA

09:45-10:10 *Engineering heme stability in recombinant hemoglobin for the development of a prototype for stable hemoglobin based oxygen carrier*
Suman Kundu, Department of Biochemistry, University of Delhi, Delhi, India

10:10-10:35 *Reengineering hemoglobin for safer synthetic oxygen therapeutics.*
Brandon Reeder, School of Life Sciences, University of Essex, United Kingdom

10:35-11:00 *Molecular oxygen activation in the cofactor-less formyl glycine generating enzyme investigated using dose-resolved X-ray crystallography*
Marina Lucic, School of Life Sciences, University of Essex, United Kingdom

11:00-11:30 Morning Refreshments and Posters, EBS 2.65 & 2.66

11:30-11:50 *Remembering Professor Andrea Mozzarelli: A Life in Structure, Dynamics, and Function of Protein*

Luca Ronda, Department of Medicine and Surgery, University of Parma, Italy

11:50-12:50 *Abstract selected speakers:*

11:50-12:05 *CRISPR/Cas9-mediated knockouts of globins 1, 2 and 3 in Drosophila melanogaster*
Ruben Petry, Molecular Genetics & Genome Analysis, iOME, Johannes-Gutenberg-University Mainz, Germany

12:05-12:20 *Mapping myoglobin expression in epithelial tissues using a cross-tissue single-cell atlas*.
Michelle Hagmaier, Institute of Organismic & Molecular Evolution, Johannes Gutenberg University Mainz

12:20-12:35 *Multi-Omics Analysis of Myoglobin Knockout Zebrafish Metabolism*.
Ciska Bakkeren, Zoophysiology, Department of Biology, Aarhus University, Aarhus, Denmark

12:35-13:30 Closing Remarks and Poster/Oral Presentation Prizes

13.30 Close of Conference

14:00-16:00 *Practical workshop: Biophysical Methods for Studying Oxygen-Binding Heme Proteins (Limited numbers, sign-up sheet at registration desk* or EBS 2.65*)*

Invited Speaker Abstracts

Anaerobic and aerobic sulfide binding to human neuroglobin: sulfheme derivatives.

Federico Sebastiani[1*], Laura Cesati[1], Mirco Meglioli[2], Maurizio Becucci[1], Marco Borsari[2], Gianantonio Battistuzzi[2], Giulietta Smulevich[1]

[1]University of Florence, Department of Chemistry "U. Schiff" DICUS, via della Lastruccia 3-13, 50019 Sesto Fiorentino (FI), Italy
[2]University of Modena and Reggio Emilia, Department of Chemical and Geological Sciences, Via Campi 103, 41125, Modena, Italy

**E-mail of the presenting author: federico.sebastiani@unifi.it*

Neuroglobin (Ngb), a neuroprotective protein present in both vertebrates and invertebrates, is predominantly expressed in neurons [1, 2]. Recently, it has been found that it is characterized by the heme rotational disorder unlike the majority of globins [3,4]. Interestingly, the heme rotational disorder has been proposed to affect not only the functional properties of the proteins, but also to be responsible for the effective mechanism of controlling ligand binding [5, 6].
Ngbs, despite their bis-histidine coordination of the heme iron, bind a number of ligands with specific physiological functions [7]. In particular, Ngb is reactive toward H_2S [8], an endogenously-synthesized signaling molecule which, being toxic at high concentration, is detoxified via different pathways [9].
Ferric neuroglobin is reduced by sulfide and catalyzes H_2S inefficient oxidative conversion to thiosulfate, while the mutation of the distal His64 residue to Ala promotes rapid binding of H_2S and its efficient catalytic conversion [8].
UV-Vis electronic absorption and resonance Raman spectroscopies have been employed to characterize the effect of sulfide binding on wild-type (WT) human Ngb. A comparative analysis of mutated human Ngbs (H64A, H64A/K67A, C46AC55A [10]) further allowed us to explore the role of key residues in ligand binding.
In addition to the formation of sulfide complexes, sulfheme adducts were found, indicating a covalent modification of one of the heme pyrrole rings, as reported for other heme proteins [11-14]. Interestingly, while the H64A and H64A/K67A variants do not form any sulfheme adduct, an opposite behaviour has been observed for the C46AC55A variant lacking the intramolecular disulfide bridge, as compared to the WT protein.
The spectroscopic data will be discussed in relation to the protein function and its active site structure.

References:

1. Vallone, Nienhaus, Brunori, Nienhaus, *Protein Sci.* **2004**, *56*, 85-92.
2. De Simone, Sbardella, Oddone, Pesce, Coletta, Ascenzi, *Cells* **2021**, *10*, 3366.
3. Milazzo, Exertier, Becucci, Freda, Montemiglio, Savino, Vallone, Smulevich, *FEBS J.* **2020**, 287, 4082-4097.
4. Sebastiani, Milazzo, Exertier, Becucci, Smulevich, *J. Raman Spectrosc.* **2021**, *52*, 1-14.
5. Exertier, Sebastiani, Gugole, Cerutti, Parisi, Montemiglio, Becucci, Viappiani, Bruno, Savino, Zamparelli, Anselmi, Abbruzzetti, Smulevich, Vallone, *ACS Chem. Biol.* **2022**, 17, 2099-2108.
6. Yamamoto, Nakashima, Kawano, Chujo, *Biochim. Biophys. Acta* **1998**, *1388*, 349-362.
7. Dewilde, Kiger, Burmester, Hankeln, Baudin-Creuza, Aerts, Marden, Caubergs, Moens, *J. Biol. Chem.* **2001**, *276*, 38949-38955.
8. Ruetz, Kumutima, Lewis, Filipovic, Lehnert, Stemmler, Banerjee, *J. Biol. Chem.* **2017**, *292*, 65126528.
9. Hildebrandt, Grieshaber, *FEBS J.* **2008**, *275*, 3352-3361.
10. Bellei, Bortolotti, Di Rocco, Borsari, Lancellotti, Ranieri, Sola, Battistuzzi, *J. Inorg. Biochem.* **2018**, 178, 70-86.
11. Rios-Gonzalez, Roman-Morales, Pietri, Lopez-Garriga, *J. Inorg. Biochem.* **2014**, *133*, 78-86.
12. Nicoletti, Thompson, Franzen, Smulevich, *J. Biol. Inorg. Chem.* **2011**, 16, 611-619.
13. Nicoletti, Comandini, Bonamore, Boechi, Boubeta, Feis, Smulevich, Boffi, *Biochemistry*, 2010, 49, 2269-2278.
Bieza, Boubeta, Feis, Smulevich, Estrin, Boechi, Bari, *Inorg. Chem.* **2015**, 54, 527-533.

Dissecting the conformational complexity of a prokaryotic heme transporter

Di Wu[1], Ahmad R Mehdipour[2,3], Franziska Finke[1], Hojjat G Goojani[4], Roan R Groh[1], Tamara N Grund[1], Thomas MB Reichhart[1], Rita Zimmermann[1], Sonja Welsch[5], Dirk Bald[4], Mark Shepherd[6], Gerhard Hummer[2,7], Schara Safarian[1,8,9]

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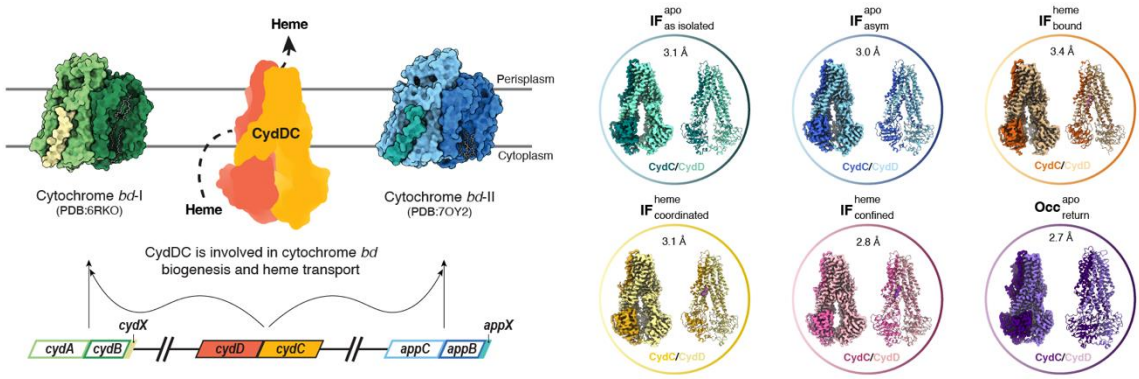
[2] Department of Theoretical Biophysics, Max Planck Institute of Biophysics, D-60438 Frankfurt/Main, Germany.
[3] Center for Molecular Modeling (CMM), Ghent University, Technologiepark 46, Zwijnaarde, 9052 Belgium.
[4] Amsterdam Institute for Life and Environment (A-LIFE), AIMMS, Faculty of Science, Vrije Universiteit Amsterdam, 1081 HZ, Amsterdam, The Netherlands.
[5] Central Electron Microscopy Facility, Max Planck Institute of Biophysics, D-60438 Frankfurt am Main, Germany.
[6] School of Biosciences, University of Kent, Canterbury CT2 7NJ, United Kingdom.
[7] Institute of Biophysics, Goethe University Frankfurt, D-60438 Frankfurt/Main, Germany.
[8] Department of Microbiology and Immunology, School of Biomedical Sciences, University of Otago, Dunedin, NZ-9054, New Zealand.
[9]: Fraunhofer Institute for Translational Medicine and Pharmacology ITMP Frankfurt, D-60438 Frankfurt/Main, Germany.

The ABC transporter CydDC is conserved in a large number of bacteria and plays a central role in the biogenesis of membrane-integrated and soluble cytochromes[1]. However, the precise physiological role of this heterodimeric ABC transporter remains unclear. In our study, we map the conformational landscape and resolve the so-far enigmatic function of this medically relevant ABC transporter. We capture this fascinating machine in action, as it powers heme transport for the assembly of redox enzymes.

In doing so, we established single-particle electron cryo-microscopy (cryo-EM) as a scalable analytical tool. We screened a large array of substrate candidates, sample conditions, and rationally designed mutant variants of CydDC. In total, we determined structures of CydDC in 23 different sample conditions at resolutions between 2.7 to 3.9 Å. This allowed us to unambiguously identify heme as the transport substrate of this transporter. On top of this, we were able to delineate the precise mechanism of substrate binding, coordination, and gating from datasets obtained under biochemically defined and turnover induced conditions. The membrane-accessible heme entry site of CydDC is primarily controlled by the conformational plasticity of CydD transmembrane helix 4, the extended cytoplasmic segment of which also couples heme confinement to a rotational movement of the CydC nucleotide-binding domain. Our cryo-EM data highlight that this signal transduction mechanism is necessary to drive conformational transitions toward occluded and outward-facing states.

Our approach underscores that systematic structural biology is able to shed light on molecular processes of many other ABC transporters whose structure and function remain elusive due to methodological limitations. Moreover, this work sets the stage for the development of novel antibacterial drugs and a new line of attack against *M. tuberculosis* and other pathogenic bacteria, where CydDC is critically important for respiratory re-wiring upon host infection.



References

[1] Shepherd, M. (2015) *Biochem. Soc. T.*, 43, 908-912.

From antibacterial strategy to modulation of hemoglobin oxygen affinity: the unexpected dual role of C35

Serena Faggiano[1,2], Sarah Hijazi[3], Francesco Marchesani[4], Marialaura Marchetti[4], Monica Cozzi[4], Valeria Buoli Comani[1], Paul Brear[5], Barbara Campanini[1,6], Luca Ronda[2,4], Mariacristina Failla[7], Eleonora Gianquinto[7], Somayeh Asghar Pour Hassan Kiyadehf[7], Barbara Rolando[7], Francesca Spyrakis[7], Carlotta Compari[1], Loretta Lazzarato[7], Omar De Bei[4], Emanuela Frangipani[3], Stefano Bettati[2,4,6]

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[5]Crystallographic X-ray Facility, Department of Biochemistry, University of Cambridge, Cambridge, United Kingdom
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[7]Department of Drug Science and Technology, University of Torino, Torino, Italy

Hemoglobin (Hb) serves as a primary iron source for several pathogenic bacteria, including *Staphylococcus aureus*, a major contributor to the global rise in antibiotic resistance (AMR). This pathogen induces red blood cell (RBC) lysis through hemolysins and captures heme from plasma free Hb by two cell wall-anchored hemophores, IsdB and IsdH, the former playing a central role in virulence (1).

To explore novel antimicrobial strategies based on iron starvation, we conducted a structure-based virtual screening campaign targeting the Hb-IsdB interface, with the goal of preventing heme extraction. This effort led to the identification of a promising compound, C35 (4-[[2-[[5-(1H-indol-3-yl)-1,3,4-oxadiazol-2-yl]sulfanyl]acetyl]amino]benzoate), which binds Hb with a dissociation constant (K_D) of 0.57 ± 0.06 μM (2).

Using both wild-type *S. aureus* Newman and an isogenic in frame-deletion mutant (Δ*isdB*), we demonstrated that C35 inhibits bacterial growth by specifically targeting the hemophore-mediated iron acquisition pathway. These results position C35 as a promising antibacterial candidate.

To elucidate the structural basis of C35 interaction with Hb, we determined the X-ray crystal structure of Hb in complex with the compound. Unexpectedly, C35 was found to bind within a cleft between the α-subunits of tetrameric Hb, stabilizing an R2 relaxed conformation of the protein. Functional assays revealed that C35 shifts of the Hb oxygen binding curve to the left, similarly to known Hb allosteric modulators such as INN298 and Voxelotor, which have been proposed for the treatment of sickle cell anemia.

Together, these findings highlight C35 as a dual-function compound, with both antimicrobial activity against *S. aureus* and the ability to allosterically modulate Hb function through non-covalent stabilization of a high-affinity state.

References

(1) Ellis-Guardiola K, et al. NEAr Transporter (NEAT) domains: unique surface displayed heme chaperones that enable Gram-positive bacteria to capture heme-iron from hemoglobin. Front. Microbiol. 2021 Jan 6;11:607679. doi:10.3389/fmicb.2020.607679

(2) Cozzi M, et al. Identification of small molecules affecting the interaction between human hemoglobin and *Staphylococcus aureus* IsdB hemophore. Sci Rep. 2024 Apr 9;14(1):8272. doi: 10.1038/s41598-024-55931-8

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Anaerobic heme catabolism in three bacterial pathogens

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The anaerobe, *Fusobacterium nucleatum*, is an opportunistic oral pathogen that is associated with colorectal cancer. *Escherichia coli* O157:H7 and *Vibrio cholera*, two facultative anaerobes, cause severe gastrointestinal illness. The ability of these organisms to establish infection at anoxic site depends on the acquisition of iron. To meet this nutritional requirement, all three of these organisms possess heme uptake/utilization operons, designated *chu* (*E. coli* O157:H7), *hmu* (*F. nucleatum*), and *hut* (*V. cholera*), that enable the organisms to acquire heme, the most abundant source of iron in the human host. Once heme enters the cell, the porphyrin ring is broken open in an O_2-independent reaction that is catalyzed by anaerobilin synthase, a radical S-adenosylmethionine-dependent enzyme. The reaction generates a linear tetrapyrrole, termed anaerobilin and releases iron that can be used for other cellular processes. Anaerobilin is cytotoxic due to its high level of conjugation and a reactive methylene group. To protect itself from the cytotoxicity of labile heme and anaerobilin, *F. nucleatum* encodes HmuF, which binds tightly to heme and traffics it to anaerobilin synthase for degradation. HmuF then catalyzes a four-electron reduction of anaerobilin, removing conjugation and the reactive methylene group. In *E. coli* O157:H7, two structurally distinct proteins, ChuS and ChuY, fulfill the function of HmuF. Strikingly, *V. cholera* does not contain a homolog for ChuS, ChuY or HmuF, raising questions about how the organism protects itself during heme degradation. We showed that HutZ of *V. cholera* binds tightly to heme (thereby preventing labile heme toxicity) and sequesters it from HutW, preventing heme degradation. Counterintuitively, *V. cholera* seemingly avoids anaerobilin toxicity by simply preventing its formation. In sum, structurally distinct *chu*, *hmu*, and *hut* operons have functionally converged to protect the cell from anaerobilin accumulation and heme cytotoxicity.

The high diversity of oxygen reactivity in bacterial terminal oxidases

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Terminal oxidases are critical for aerobic respiratory chains of prokaryotes and eukaryotes, responsible for the final step in the electron transport chain. These membrane proteins catalyse the transfer of electrons from reduced electron carriers (such as cytochrome c or quinols) to the terminal electron acceptor, molecular oxygen, thereby reducing it to water. They play a pivotal role in aerobic respiration and energy metabolism, adapting to diverse environmental and physiological needs across different organisms.[1,2]

First an overview of the electrochemical properties of terminal oxidases from different organisms will be given and their high degree of adaptivity with redox potentials spanning more than 500 mV discussed, with a special focus on bacterial oxidases. The electrocatalytic response observed by means protein film voltammetry will be described, giving insight into the rich and complex electron and proton transfer catalysed by these essential enzymes.

The superfamily of cytochrome *bd* oxidases are solely present in prokaryotes. These enzymes play a crucial role in protection against oxidative stress, in virulence, adaptability and antibiotics resistance.[3,4] Recent structural data, electrochemical and spectroscopic studies allowed obtaining information on the oxygen reduction, coupled protonation processes, and the interaction with small molecules that rule the signaling processes in the biological cell, including NO.

1. Melin, F., Hellwig, P. ‘Redox Properties of the Membrane Proteins from the Respiratory Chain' (2020), Chem. Rev., 120, 10244–10297.
2. Hellwig, P. 'The electrochemical properties of the highly diverse terminal oxidases from different organisms' (2025) Bioelectrochemistry 165, 108946.
3. Borisov, V.B. Siletsky, S.A. Paiardini, A. Hoogewijs, D. Forte, E. Giuffrè, A. Poole, R.K. ‘Bacterial Oxidases of the Cytochrome bd Family: Redox Enzymes of Unique Structure, Function, and Utility As Drug Targets’ (2020) Antioxid. Redox Signal. 34, 16, 1280-1318.
4. Safarian, S.; Hahn, A.; Mills, D.J.; Radloff, M.; Eisinger, M.L.; Nikolaev, A.; Meier-Credo, J.; Melin, F.; Miyoshi, H., Gennis, R.B.; Sakamoto, J.; Langer, J.D.; Hellwig, P.; Kühlbrandt, W.; Michel, H. ‘Active site rearrangement and structural divergence in prokaryotic respiratory oxidases’ (2019), Science, 366, 100-104.

Targeting aerobic respiratory chains to combat bacterial pathogens

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Cytochrome *bd* complexes are terminal respiratory oxidases found exclusively in the aerobic respiratory chains of prokaryotes that generate a proton motive force by coupling quinol oxidation to the reduction of dioxygen. Previous work has demonstrated that cytochrome *bd* complexes are important during infection for a variety of bacterial pathogens, demonstrating their potential as drug targets. While the core machinery of cytochrome *bd* is generally well-conserved, interesting variations exist that will influence drug binding. Hence, it is important to have a range of tools to investigate drug binding to purified protein complexes as well as in membrane environments.

We have cloned cytochrome *bd* complexes from a range of bacterial pathogens and have expressed them in an *E. coli* strain that lacks all respiratory oxidases (Portnoy et al. 2010). It was possible to purify several of these complexes and interrogate haem cofactor assembly via difference spectroscopy and to monitor kinetic activities spectroscopically. Our new high-throughput assay monitors spectroscopic changes during quinol oxidation, and this approach was validated by comparing kinetic measurements to data obtained using electrodes and fluorimetric methods that detect oxygen depletion. Furthermore, previous work in the Shepherd lab has reported steroid drugs as potent inhibitors of cytochrome *bd* complexes (Henry *et al.* 2024), although the modes of binding have not previously been investigated using laboratory approaches. Apparent K_m values were measured with respect to quinol, and inhibition kinetics experiments were performed to investigate the binding location for the steroid drug quinestrol. The methodological advances herein will be of interest to those working on any quinol-oxidising respiratory complex, and we intend to exploit this approach for broader drug screening of cytochrome *bd* complexes, a topic of much interest over the past decade.

References

Portnoy, V. A. *et al*. (2010) Appl. Environ. Microbiol. 76, 6529–6540

Henry, S. A. *et al.* (2024) J. Infect. Dis. **230**, e149–e158

Conformational analysis of human and crocodilian Hb by cryo-electron microscopy

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With a molecular weight of around 64 kDa, hemoglobin (Hb) remains a small protein for cryo-EM analysis, but recent improvements in hardware and software have made it possible. Models of oxidised (met) Hb have been determined by various groups using cryo-EM, with commercially available lyophilised human Hb. One group[1] reported about 20% of the sample was in the form of $\alpha\beta$ dimers, rather than the expected heterotetrameric form, which gives some indication of the sample quality. In Yokohama we have used fresh Hb samples prepared from human blood. Collaborators in Aarhus and Nebraska also provided Hb purified from the blood of *Alligator mississipiensis*, the American alligator. Bicarbonate ions strongly reduce the oxygen affinity of adult crocodilian Hbs, an effect believed to contribute to the lengthy diving times (of several hours) of these animals. 30 years ago, mutational studies of human Hb managed to transplant this unusual bicarbonate effect to human HbA, but the precise details of the interaction remained unknown until last year. In 2024 the cryo-EM structure of deoxy alligator Hb bound to bicarbonate ions showed the molecular interactions formed between the protein and the allosteric effector.[2] Both human and alligator Hbs were also studied in the liganded form, so that the allosteric changes of both human and alligator Hb could be described in the report.

Human Hb was believed from early crystallographic studies to form only two conformations, a deoxy T state and a liganded R state, but in the 1990s the group of Arnone described a new liganded structure called R2.[3] Liganded Hb apparently explores a considerable conformational space, much more so than deoxy-Hb. Since cryo-EM analysis does not require crystallisation, it potentially provides a more realistic view of the true conformational state (and variability) of Hb in the liganded form. The initial cryo-EM analysis of the two proteins suggested that although carbonmonoxy human Hb prefers an R2-like conformation, the CO-bound alligator protein was found in a conformation closer to the classical R structure. Re-analysis was performed specifically to examine other conformations present in the images, and showed two additional structures, one for human and one for alligator protein. While it was found that liganded human Hb does form the R conformation to a large degree, the alligator protein does not form R2. Instead it shows a novel quaternary structure that we describe as "T-like", which is quite different from R or R2. This form may be the result of conserved interactions being disrupted by the introduction of the bicarbonate binding site.

References

1) Herzik MA., Wu, M and Lander, GC. (2019) Nat. Comm. 10(1)1032
2) Takahashi K, Lee Y, Fago A, Bautista NM, Storz JF, Kawamoto A, Kurisu G, Nishizawa T, Tame JRH. (2024) Nat. Comm. 15(1)1032
3) Silva MM, Rogers PH, Arnone A. (1992) J Biol Chem. 267:17248-56

Ngb function in Zebrafish – lessons learned from a knockout model

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Neuroglobin (Ngb) is an ancient member of the globin family that has been extensively studied in mammals since its discovery in 2000. According to the majority of studies, mammalian Ngb plays a role in cellular oxygen metabolism and neuroprotection against reactive oxygen species (ROS) and, during hypoxia, in apoptosis regulation and mitochondrial function. Evidence suggests that Ngb contributes to the stress tolerance in hypoxia-adapted vertebrates like the naked mole rat and goldfish. Using CRISPR/Cas9, we generated a Ngb knockout (KO) in zebrafish, a tropical teleost known for its tolerance to hypoxia and temperature fluctuations. Hypoxia and temperature, two abiotic factors affecting fish energy metabolism, were employed to investigate the *in vivo* function of Ngb. Our study is one of the first *in vivo* reports to address the function of Ngb in the adult zebrafish via analysis of CRISPR/Cas9-mediated knockouts. We performed transcriptome analyses of brain tissue and compared wild-type and KO tissues. Our work provides unique insights into the diverged functions between the mammalian and zebrafish *neuroglobin* and highlights the usefulness of zebrafish as a model organism for functional genomic research.

Convergent reductions in hemoglobin buffering power in lineages of small, high-metabolic rate birds and mammals: implications for O_2 delivery

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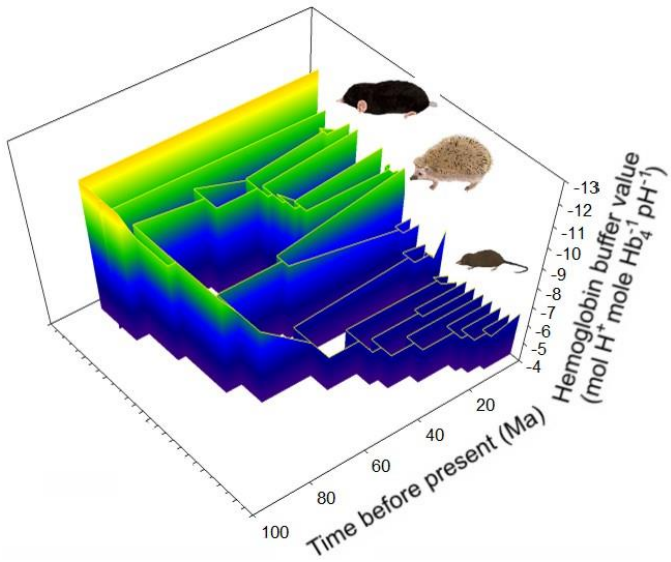
The mass-specific O_2 requirements of the smallest (2 g) endothermic birds and mammals are a remarkable ~375 times higher than those of 10,000 kg elephants; accordingly, their tissues require much higher rates of O_2 delivery *at rest* than those of larger species *during vigorous exercise*. To help meet these demands, numerous physiological (*e.g.*, blood with a high hemoglobin concentration) and molecular modifications to hemoglobin (*e.g.*, reduced O_2 affinity and a high Bohr effect) have been proposed. Here we examined another potential hemoglobin specialization, namely reductions in hemoglobin buffering power (β_{Hb}; primarily arising from reductions in titratable histidine content). Briefly, we hypothesized that a low β_{Hb} should produce an exaggerated reduction in red blood cell pH for a given acid (CO_2) load, which would magnify the Bohr effect (*i.e.*, cause a stronger rightward shift of the oxygen equilibrium curve) to augment tissue O_2 offloading. To test this hypothesis, we first calculated the predicted buffering power of Hb (mol H^+ · mol Hb_4^{-1} · pH^{-1} at 37°C, pH 7.2) for 374 avian and 489 mammalian species from the primary structures of their component globin chains, which we show to be strongly positively correlated with measured β_{Hb} over a range of avian (n=11) and mammal (n=7) species. Consistent with our expectations, strong (~30-50%) independent reductions in βHb were observed in five active small-bodied bird and mammal lineages (hummingbirds, passerines, shrews, bats, and dasyurid (carnivorous) marsupials). Notably, convergent replacements of histidine residues at five positions that contribute strongly to β_{Hb} but have minimal influence on the Bohr effect largely underlie reductions in βHb in these high mass-specific metabolic rate clades. Theoretical modelling employing measured βHb in shrews (which is 46% lower than the βHb of adult human hemoglobin) suggests that although this trait alone nearly doubles the CO_2 Bohr effect, overall O_2 offloading is only increased by ~2-4% per transit through the systemic capillaries. However, the speed of diffusion will also increase via increased systemic capillary plasma-to-tissue O_2 partial pressure gradients relative to larger-bodied species. Finally, since the red blood cells of embryonic birds and most fetal mammals contain the same hemoglobin isoforms as found in adults, evolutionary reductions in βHb are also expected to increase maternal-fetal O_2 exchange in mammals via the well-known 'double Bohr effect', while aiding systemic O_2 offloading in both prenatal birds and mammals. Accordingly, evolutionary reductions in β_{Hb} represent a previously overlooked mechanism to aid O_2 delivery in small, highly active endothermic species.



Convergent reductions in hemoglobin buffering power in lineages of small, high-metabolic rate birds and mammals: implications for CO_2 elimination

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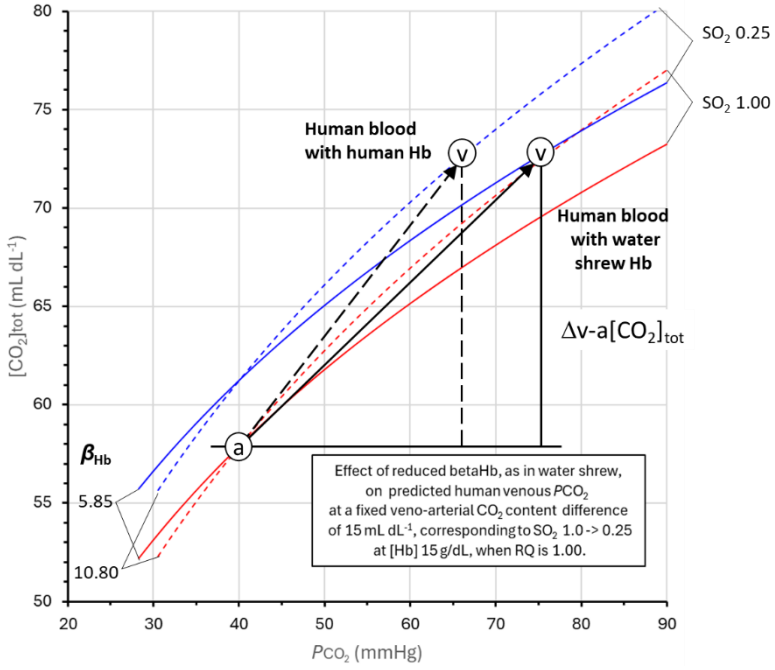
The smallest mammalian and avian species are faced with the dual challenge of possessing the highest mass-specific rates of O_2 uptake and CO_2 elimination coupled with the shortest capillary transit times for gas exchange. We've previously demonstrated significant (~30-50%) reductions in hemoglobin buffer power (βHb) in five lineages of small, high mass-specific metabolic rate endotherms (hummingbirds, passerines, shrews, bats, and dasyurid (carnivorous) marsupials) that, together with relatively high Bohr effects (acidity induced reduction of hemoglobin-O_2 affinity), cooperativity coefficient's, and low blood O_2 affinities, help maximize tissue O_2 delivery. However, as rates of O_2 consumption and CO_2 production are mechanistically linked via the respiratory quotient, any increase in O_2 uptake and delivery must be met by commensurate increases in CO_2 transport and excretion. This metabolic waste product is predominantly carried in the venous plasma in the form of bicarbonate ions (HCO_3^-) which markedly increases the overall CO_2 carrying capacity of blood. Briefly, most generated CO_2 first diffuses into the RBCs where a large fraction is converted into HCO_3^- and H^+ by carbonic anhydrase. The rapid hydration of CO_2 is sustained by the buffering of protons by T-state hemoglobin and concomitant transmembrane exchange of HCO_3^- for chloride ions (Cl^-)—termed the chloride shift—by the band 3 anion transporter. However, the subsequent diffusion of CO_2 into the alveolar space is rate limited by the reciprocal transfer of these ions by band 3, potentially leading to exchange disequilibria between the lung capillaries and alveoli during strenuous exercise. Since small high metabolic endotherms should be particularly predisposed to such CO_2 exchange limitations given their disproportionally higher mass-specific rates of CO_2 production and more rapid capillary transit times than larger species, we modelled the effect of a 46% reduction in βHb in shrews (relative to adult human hemoglobin) on blood CO_2 transport. Our results demonstrate that—by lowering the amount of HCO_3^- formed at a given red cell CO_2 partial pressure—evolutionary reductions in βHb within lineages of small, high metabolic rate birds and mammals functions to markedly elevate venous plasma to alveolar CO_2 partial pressures gradients (by up to 32%, e.g. from 67 mm Hg to 75 mm Hg compared to 40 mmHg in alveoli), thereby expediting the elimination of this metabolic by-product via an elevated blood-to-alveolar CO_2 transfer gradient. Accordingly, by promoting an elevated lung microcirculatory diffusion rate of CO_2, a low βHb appears to represent an important evolutionary innovation to maximize O_2 consumption rates during exercise by members of these five high mass-specific metabolic rate lineages.



Phytoglobins - some new insights into their structure and biological function

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Phytoglobins (Pgbs) are plant-originating heme proteins belonging to the globin superfamily. Here we present a comprehensive and integrated biophysical study of three nsHbs in sugar beet (*Beta vulgaris*). We have analyzed their reactions with oxygen, CO, NO, and how these are regulated by the coordinating distal histidine. We also evaluate their capacity to carry out nitrite reductase (NiR) and NO dioxygenase (NOD)-like functions. Pgbs predominantly adopt a hexacoordinated configuration, which results in exceptionally strong binding to gaseous ligands like NO and CO. These distinctive ligand-binding properties often makes it difficult to determine their binding affinities through standard analytical approaches. Assaying binding affinities between proteins and gaseous species in solution is a fundamental task in biochemistry, with implications for understanding enzymatic catalysis, signaling pathways, and protein functionality. In this presentation, we demonstrate a streamlined microscale method employing fluorophore-labelled proteins in gas-saturated microscale capillaries. The assay significantly reduces protein requirements and experimental complexity, providing efficient and accurate binding affinity measurements.

On a more detailed protein level, it is easily realised that Pgbs carry a unique conserved cysteine residue, the role of which is only poorly understood. We have therefore investigated the functional and structural role of cysteine in BvPgb1.2, a Class 1 Pgb from sugar beet (*Beta vulgaris*), by constructing an alanine-substituted mutant (Cys86Ala). The substitution had little impact on structure, dimerization, and heme loss as determined by X-ray crystallography, NMR and SAXS measurements. However, several other important biochemical properties were modified. Particularly, the analysis of conformational dynamics provided a better understanding of the biological functional of the protein.

Pgbs often act in concert with other proteins and biomolecules *in vivo*. An interesting example is its strong interaction with the protein glucose-6-phosphate dehydrogenase (G6PDH). Here we propose that G6PDH facilitate the transfer and insertion of the heme group to Pgb. Similarly, phytoglobins have a strong affinity for DNA, which may implicate that these globins can influence a more general transcriptional control.

The functional implications of these studies will be discussed in light of the differential expression of the phytoglobins *in vivo* and the present knowledge on seed germination, and sugar translocation. The results expand our knowledge and provide an overall view on the redox chemistry of Pgbs.

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Heme-based dioxygenases: Structure, function and dynamics

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Tryptophan dioxygenase (TDO) and indoleamine 2,3 dioxygenase (IDO) belong to a unique class of hemebased enzymes (Fig. 1) that insert dioxygen into the essential amino acid, L-tryptophan (Trp), to generate N-formylkynurenine (NFK), a critical metabolite in the kynurenine pathway. Recently, the two dioxygenases were recognized as pivotal cancer immunotherapeutic drug targets, which triggered a great deal of drug discovery targeting them. The advancement of the field is, however, hampered by the poor understanding of the structural properties of the two enzymes and the mechanisms by which the structures dictate their functions. In this talk, I will summarize recent findings centered on the structure, function, and dynamics of the human isoforms of the two enzymes.

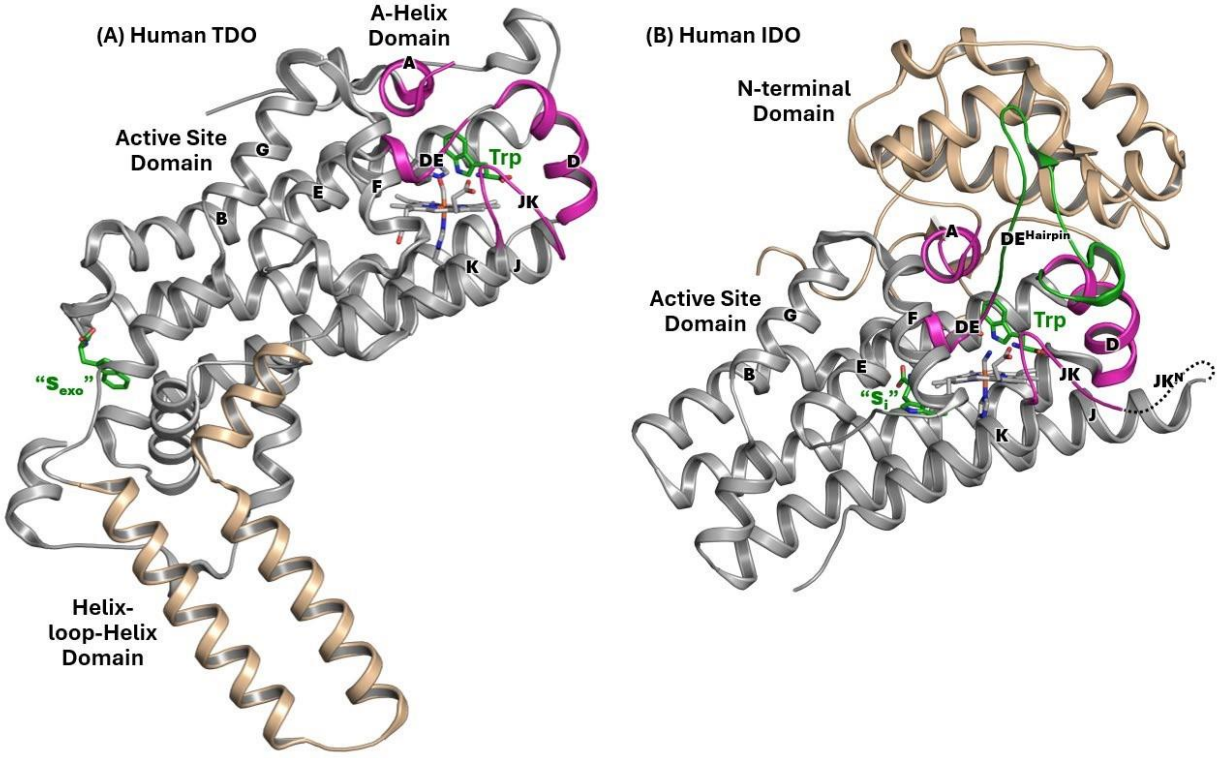

Fig. 1. Structural comparison of human TDO and IDO. TDO is a homo tetrameric protein, made by a dimer of dimers. Each dimer is stabilized by domain swapping of a small N-terminal A-Helix. Each monomer, shown in (A), contains two domains: (i) the active site domain, which binds a heme and a substrate, Trp, and (ii) the Helix-loop-Helix domain, extending out if it, The A-Helix forming the roof of the active site is from the neighboring monomer. IDO, in contrast, is a monomeric protein. As shown in (B), it also contains two domains: (i) the active site domain, which binds a heme and a substrate Trp, and (ii) a N-terminal domain sitting on top of it. Although the active sites of the two proteins share high structural similarities, TDO and IDO exhibit several distinct structural features. In particular, the Helix-loop-Helix domain present in TDO is absent in IDO; conversely, the N-terminal domain, DE-hairpin, and the N-terminal extension of the JK-Loop (JK-Loop^N, shown as the dotted line) present in IDO are absent in TDO. Furthermore, TDO binds a second substrate Trp in an exosite (S_{exo}), which is ~40 Å away from the active site. Binding Trp to the S_{exo} site controls the cellular lifetime of the protein by regulating the degradation of the protein *via* the ubiquitin-linked proteasome pathway. IDO also binds a second Trp, but it is in a unique inhibitory site (S_i) on the proximal side of the heme. Binding Trp to the S_i site leads to the inhibition of the Trp dioxygenation activity.

Reference: “Heme-based dioxygenases: Structure, function and dynamics." Geeraerts Z, Ishigami I, Gao Y, Yeh SR. J Inorg Biochem. 2024 Dec;261:112707. doi: 10.1016/j.jinorgbio.2024.112707.

Structural insights into the functional properties of cytochrome c oxidase

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Cytochrome *c* oxidase (C*c*O) is the membrane-bound terminal enzyme in the electron transfer chain in all organisms that utilize oxygen to generate energy. It is responsible for >90% of the oxygen utilization in the biosphere. In mitochondria, C*c*O catalyzes the four-electron reduction of O_2 to H_2O, thereby maintaining electron flow for oxidative phosphorylation. At the same time, it harnesses the oxygen reduction energy to translocate four protons across the mitochondrial membrane, thereby augmenting the electrochemical proton gradient required for the generation of ATP by ATP-synthase. A great deal of research has been conducted to comprehend the molecular properties of C*c*O. However, the mechanism by which the oxygen reduction reaction is coupled to proton translocation remains poorly understood. Here, we present the chemical properties of a variety of key oxygen intermediates of bovine C*c*O revealed by time-resolved resonance Raman spectroscopy and the structural features of the enzyme uncovered by serial femtosecond crystallography, an innovative technique that allows structural determination at room temperature without radiation damage by using femtosecond pulses from an X-ray free electron laser for diffraction from C*c*O microcrystals.[1,2] These data support a proton translocation mechanism in mammalian C*c*O which is distinct from that in bacterial oxidases.[3]

References

1 Ishigami, I. *et al.* Snapshot of an Oxygen Intermediate in the Catalytic Reaction of Cytochrome c Oxidase. *Proc Nat Acad Sci (USA)* **116**, 3572-3577 (2019).

2 Ishigami, I. *et al.* Structural insights into functional properties of the oxidized form of cytochrome c oxidase. *Nature communications* **14**, 5752 (2023).

3 Rousseau, D. L., Ishigami, I. & Yeh, S. R. Structural and functional mechanisms of cytochrome c oxidase. *Journal of inorganic biochemistry* **262**, 112730 (2025).

Cytochrome b_5 reductase 4 efficiently reduces Neuroglobin and Cytoglobin

Anthony W. DeMartino [1,2], Onaje Cunningham [1], Efthimios Deligiannidis [1], Stefan Kaliszuk [1], Saumika Mulluri [1], Deandra Cassel [1] and Jesús Tejero [1,3,4,5]

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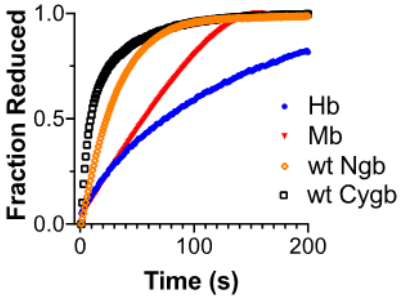
Cytoglobin and Neuroglobin are heme-containing proteins expressed in most vertebrates, including mammals, with still not completely defined functions. These proteins are evolutionarily related to the ubiquitous oxygen carriers hemoglobin and myoglobin, but present a bis-histidine, hexacoordinated heme iron, unlike the five-coordinated heme iron in hemoglobin/myoglobin. These differences reflect in their heme binding properties and reactivity. Importantly, cytoglobin and neuroglobin do not appear to function as oxygen carriers, but seem to participate in different processes related, at least in part, to reactive oxygen species detoxification and/or signaling.

Most of the putative functions of cytoglobin/neuroglobin, such as oxygen binding or nitric oxide dioxygenation, rely on the heme iron being in the ferrous (Fe^{2+}) oxidation state. It is well established that the ferrous cytoglobin and neuroglobin have high oxygen affinities and autoxidation rates much faster than those of hemoglobin and myoglobin. This indicates that in the absence of a reducing agent, both proteins will be present in cells in a ferric (Fe^{3+}) resting state, unable to participate in most of their presumed roles. Therefore, it is very possible that reducing systems are active in the cell to maintain both proteins in the ferrous state.

In previous work we have shown that the cytochrome b_5 reductase 3/ cytochrome b_5 system, the canonical reductase of hemoglobin and myoglobin, can reduce cytoglobin at very fast rates, consistent with a possible physiological role. Those observations have been confirmed in vivo. However, this reducing system is unable to reduce neuroglobin, which to date lacks a validated, physiologically feasible reducing system.

Five different isoforms of cytochrome b_5 reductase are present in humans. Cytochrome b_5 reductase isoforms 1, 2, 3, and 5 use cytochrome b_5 as electron transfer partner. However, cytochrome b_5 reductase 4 is a fusion cytochrome b_5 reductase-cytochrome b_5 protein, also expressed ubiquitously in mammals. We have studied the interaction of cytochrome b_5 reductase 4 with cytoglobin and neuroglobin and found that cytochrome b_5 reductase 4 can reduce cytoglobin at rates similar to those observed with cytochrome b_5 reductase 3/ cytochrome b_5. Remarkably, it can also reduce neuroglobin efficiently. Studying different surface mutations of cytoglobin and neuroglobin we note that some cytoglobin mutations, in particular R84E and K116E do decrease reduction rates by more than 10-fold, whereas surface mutations in neuroglobin that were shown to impair cytochrome *c* interaction (E60K/D73K/E87K) show minimal effect on the reduction rates. We conclude that cytochrome b_5 reductase 4 can supplement cytochrome b_5 reductase 3/ cytochrome b_5 roles for cytoglobin reduction in vivo and is a strong candidate for a physiological role as neuroglobin reductase.



Time course of the reduction of wild-type heme proteins (≈10 μM) by 500 nM Cytochrome b_5 reductase 4 in the presence of 200μM NADH.

***Candida albicans* Utilises Methaemoglobin to Build Ultra-Drug Resistant Polymicrobial Biofilms**

Ed Deshmukh-Reeves and Campbell Gourlay

Kent Fungal Group, School of Natural Sciences, University of Kent

The growth of drug resistant poly-microbial biofilms represents a major clinical problem that underpins recurrent infection and failed therapy. Here we show how the presence of Methaemoglobin, (MetHb), which forms when the iron component in haemoglobin is oxidised from the ferrous (Fe2+) to the ferric (Fe3+), state is bound by *C. albcians* into dense biofilms structures that show remarkable drug resistant properties. Our analysis suggests that *C. albicans* is able to lyse red blood cells to liberate MetHb as structural component for biofilm establishment, as well as using it as a source of nutrition. The *C. albicans*/MetHb aggregate biofilms that form show increased virulence most likely linked to accelerated hyphal extension. Furthermore, we show that the presence of *C. albicans/*MetHb increases the rate of *S. aureus* and *P. aeruginosa* incorporation in biofilms. As MetHb levels are increased within a number of disease pathologies, including sepsis, poly-microbial/MetHb aggregate biofilms may represent an as yet unexplored structure that supports persistence, metastatic infection and poor patient outcome.

Cytoglobin in Hepatic Stellate Cells Plays an Anti-fibrotic Role in Chronic Liver Injury

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Cytoglobin (CYGB) was originally identified in primary cultured rat hepatic stellate cells (HSCs) through a proteomics approach. CYGB is a hexacoordinated globin that, like other globins, binds to gaseous molecules such as oxygen, nitric oxide (NO), and carbon dioxide. It also exhibits NO dioxygenase and superoxide dismutase activities, functioning as an antioxidant. HSCs, which store vitamin A, are located in the liver sinusoids within the space of Disse, situated between hepatocytes and liver sinusoidal endothelial cells. During hepatitis, HSCs become “activated” and begin producing type I collagen and TGF-β, contributing to fibrosis, cirrhosis, and eventually liver cancer.

CYGB is abundantly expressed in HSCs in normal human liver tissue but is notably diminished in HSCs within cirrhotic liver tissue. This observation raises important questions about CYGB’s role in the pathology of chronic liver injury. To explore this, we developed and analyzed several chronic liver injury models in CYGB knockout (KO) mice. Across all models, CYGB deficiency led to enhanced HSC activation, promoting liver fibrosis and hepatocarcinogenesis. In contrast, CYGB overexpression attenuated these pathological processes.

In CYGB-deficient HSCs, the NO dioxygenase activity is lost, resulting in delayed NO degradation. This prolongs NO signaling and allows NO to diffuse into neighboring hepatocytes, where it disrupts mitochondrial complex IV activity. The resulting increase in oxidative stress may lead to oxidative DNA damage in hepatocytes. One mechanism underlying the downregulation of CYGB during chronic liver injury involves TGF-β signaling: activated pSMAD2 recruits the M1 repressor isoform of SP3 to the human CYGB promoter (nucleotide positions +2 to +13 from the transcription start site), thereby suppressing CYGB transcription.

These findings suggest that maintaining CYGB expression in HSCs may help protect liver tissue and inhibit fibrosis progression. Interestingly, we discovered that recombinant human CYGB is spontaneously taken up by cultured HSCs, significantly suppressing collagen production in a CYGB dose-dependent manner. In vivo experiments further revealed that intravenously administered recombinant CYGB preferentially accumulates around liver sinusoids and effectively suppresses liver fibrosis in a thioacetamide (TAA)-induced chronic liver injury model.

Based on these results, we are currently investigating the potential of CYGB-based peptide therapy as a treatment for chronic liver disease. Additionally, hypermethylation of the CYGB promoter region has been observed in human liver cancer tissues, correlating with reduced CYGB expression and suggesting a possible link between CYGB downregulation and hepatocarcinogenesis.

Targeting Nitric Oxide-Driven Cancer Progression: Hemin Derivatives Suppress Migration and Angiogenesis via Redox Pathway Modulation

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Introduction: Triple-negative breast cancer (TNBC) is a highly aggressive subtype with limited treatment options.[1] Nitric oxide (•NO), a key mediator in the tumour microenvironment, promotes metastasis, angiogenesis, and resistance to therapy.[2,3] Although iNOS inhibitors have been explored, their systemic toxicity limits their application. Hemin (Fe(III)-protoporphyrin IX) selectively binds •NO, but its instability and aggregation hinder its therapeutic use.[4] The objectives of this study were to: (i) synthesize hemin derivatives (H-Styr, H-Tyra, H-Tyros); (ii) assess their solubility, stability, protein interaction, and •NO selectivity; and (iii) evaluate their anti-migratory and anti-angiogenic effects through redox modulation.

Materials and Methods: Hemin derivatives were synthesized via metathesis and carbodiimide coupling, and characterized as previously reported.[5,6] •NO-binding was assessed via real-time electrochemical sensing. Biological studies were conducted in MDA-MB-231 and iNOS-overexpressing HCC1806 TNBC cell lines to assess cytocompatibility, intracellular •NO levels (DAF-FM), and cell migration (scratch and transwell assays). VEGF secretion, cytokine release, and glycoprotein changes were quantified using ELISA, arrays, and lectin staining, respectively. Mitochondrial function was assessed using a Seahorse XFp Analyzer, and Western blotting was used to evaluate HOX-1 and protein nitration. Zebrafish vasodilation assays were used to assess the in vivo anti-angiogenic activity.

Results and Discussion: All three hemin derivatives were successfully synthesized and characterized using UV-Vis, FTIR, LC-MS, and ^{1}H NMR. DFT studies revealed enhanced π-conjugation and electron delocalization, which correlated with increased •NO affinity and oxidative stability. Electrochemical assays confirmed strong •NO-scavenging activity, with H-Tyros exhibiting the highest reactivity.
Intracellular assays confirmed reduced •NO levels in TNBC cells treated with 4–8 μM of each compound. Migration assays demonstrated that H-Styr and H-Tyros significantly inhibited •NO-induced motility (>60% reduction), accompanied by decreased VEGF secretion and alterations in glycoprotein expression. Seahorse analysis revealed decreased mitochondrial oxygen consumption rates, suggesting redoxmediated metabolic reprogramming in the cells.
Western blotting showed alterations in HOX-1 and protein nitration levels, indicating interference with •NOrelated signalling cascades, with variations in the binding affinity to BSA. Importantly, in zebrafish models, the derivatives reversed •NO-induced vasodilation, highlighting their anti-angiogenic activity in vivo.

Conclusion: These hemin derivatives selectively modulate •NO signalling and inhibit TNBC progression through redox interference. These multifunctional properties offer a promising platform for •NO-targeted therapeutics in aggressive cancers.

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References

1. Penault-Llorca, F. & Viale, G. *Ann. Oncol.* **23**, (2012).
2. Alsharabasy, A. M. *et al. Transl. Sci.* **6**, 1416–1432 (2023).
3. Fukumura D, Kashiwagi S, Jain RK. *Nat Rev Cancer*. **6**, 521 (2006).
4. Alsharabasy, A. M., Pandit, A. & Farràs, P. *Adv. Mater.* **33**, 2003883 (2021).
5. Pandit, A. *et al. ChemRxiv* (2022).
6. Alsharabasy, A. M. *et al*. *Small Sci.* **4**, 2400237 (2024).

Stefano Bruno -Awaiting submission of Abstract

Androglobin in basal metazoans: ancient functional association with cilia

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Globins are a diverse family of heme-containing proteins traditionally associated with oxygen transport and storage. The structurally unique member of this family, androglobin (Adgb), is a large protein that includes a permuted globin domain interrupted by a calmodulin-binding motif and a putative protease domain. Its chimeric nature combines oxygen and calcium sensing with proteolysis, making it an intriguing candidate for signalling cascades under e.g. changing oxygen conditions. Adgb is conserved across nearly all metazoan phyla, suggesting an ancient evolutionary origin and potentially fundamental cellular function. In mammals, Adgb is expressed in testes and multi-ciliated cells, and its loss impairs fertility and structure of motile cilia. Here, we investigate whether Adgb's expression and ciliary association are conserved in basal metazoans and unicellular relatives. Using transcriptomic datasets from model species of all metazoan clades, we demonstrate that Adgb is consistently expressed in male germ cells and ciliated cell types. Adgb expression is also found in cell types that are considered to be lineage-specific, such as choanocytes of sponges. In addition, Adgb transcription is regulated by the ciliary transcription factor RFXa in the choanoflagellate *Salpingoeca rosetta*, indicating that Adgb is an ancient core component of the ciliome. These findings point to a deep evolutionary link between Adgb and ciliated or flagellated cells and support its use as a marker for such cell types across metazoan evolution. Our identification of new Adgb-like sequences in flagellated unicellular eukaryotes suggests an even earlier origin, possibly tracing back to the last eukaryotic common ancestor. Structural predictions performed with AlphaFold2 showed high concordance between Adgb proteins and simultaneously revealed intriguing innovations in the metazoan lineage, which could have contributed towards the shift from minor ciliary motility cofactor towards essential fertility component in mammals. Currently, we focus on elucidating the exact contribution of the different domains to the proposed ciliary function.

Jian Cui-Awaiting submission of Abstract

Paola Corti-Awaiting submission of Abstract

Illuminating Heme Loss from Hemoglobin: New Fluorescent Tools to Track Heme-Iron Flow During Infection

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Introduction
Hemoglobin (Hb) is best known for transporting oxygen, a function enabled by its iron-containing heme groups. These heme units also constitute the body's largest iron reservoir, making Hb a prime target for bacterial pathogens during infection. Many microbes exploit Hb by scavenging intact protein or free heme, particularly from its β subunits. This heme piracy is essential for bacterial survival and simultaneously disrupts host oxygen delivery. However, directly tracking heme loss from Hb has been technically challenging due to the limited sensitivity and temporal resolution of existing methods. In this presentation, I will discuss the development of novel fluorogenic heme-transfer sensors and their use in uncovering how pathogens scavenge heme-iron from human Hb.

Methods
To overcome these challenges, we developed a series of fluorescent hemoglobin sensors—Hb56TAM, Hb23TAM, and Hb78TAM. These constructs use site-specific attachment of tetramethylrhodamine (TAMRA) fluorophores to detect heme release via changes in fluorescence quenching. The sensors enable real-time, titrimetric, and kinetic analysis of heme transfer from Hb to bacterial cells or heme-binding proteins. Additionally, we designed CR2-EGFP sensors to monitor heme flow between bacterial hemoproteins that mediate heme scavenging. These sensors detect heme occupancy through changes in the fluorescence of the fused Enhanced Green Fluorescent Protein (J Inorg Biochem. 2023;249:112368).

Results/Discussion
We evaluated both sensor types for their ability to track heme flow from human Hb to bacterial receptors. The Hb-TAMRA sensors reliably detected heme dissociation events, showing fluorescence increases upon heme transfer—even in complex media. Each variant revealed subunit-specific differences in heme release dynamics. Using the CR2-EGFP sensors, we found that secreted and cell wall–associated hemoproteins from Actinobacteria interact to rapidly relay heme, enhancing iron acquisition from Hb. These findings suggest that pathogens preferentially target the more labile β subunits of Hb and exploit cooperative hemoprotein networks to efficiently capture host iron.

Conclusions
These fluorescent tools provide a sensitive and effective method for studying heme release from hemoglobin and its capture by pathogens. By enabling real-time tracking under near-physiological conditions, they offer new insights into iron competition during infection and lay the groundwork for future studies on hemoglobin dynamics and bacterial interference.

Engineering heme stability in recombinant hemoglobin for the development of a prototype for stable hemoglobin based oxygen carrier

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Artificial blood substitutes have long been the holy grail of protein science. Recombinant hemoglobins (rHbs) are promising candidates due to their extended shelf life and reduced risk of immunological or infectious disease related complications. However, a major limitation of current rHb-based oxygen carriers (rHBOCs) is rapid heme dissociation, which can lead to toxicity and loss of function. We aim to enhance heme retention in the genetically cross-linked rHb0.1, which contains polypeptide stabilizing mutations and a glycine linker to prevent αβ dimer dissociation. Inspired by nature's design of covalent and co-axial heme-protein interactions, we introduced a specific point mutation near the heme vinyl group to improve heme stabilization, following the success in creating an unprecedented heme stability in myoglobin based on this concept. The resulting mutant showed a 3–4-fold increase in heme retention compared to the wild-type rHb0.1, with improved thermal stability and a slightly lower autooxidation rate. Additionally, it exhibited oxygen affinity similar to red blood cell Hb in the presence of allosteric effectors. These findings demonstrate that targeted mutagenesis can enhance heme retention without compromising function, providing a rational approach to developing stable and physiologically compatible rHBOCs.

Reengineering Hemoglobin for Safer Synthetic Oxygen Therapeutics

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Introduction: Among efforts to develop more viable Hemoglobin-Based Oxygen Carriers (HBOC), various strategies have been employed to stabilize the hemoglobin (Hb) outside of the cell with the most common method being chemical modification. However, this does not address the issue of the redox active nature of Hb. Hb can initiate oxidative reactions whenever it is separated from the environment of the erythrocyte, indicating that this redox chemistry is an inherent property of Hb and Hb-based products. Using recombinant technology to generate HBOCs provides a platform to target the inherent toxicities of Hb using genetic engineering. Although some effort has focused on the nitric oxide scavenging properties of Hb, the propensity of Hb to initiate oxidative reactions has seen much less focus.

Materials and Methods: HBOC was expressed by scalable fermentation followed by a 2-stage purification process before endotoxin removal and PEGylation. Protein stability (autoxidation, heme loss), propensity to induce oxidative damage (by lipid oxidation) and NO scavenging were closely monitored. Focused preclinical studies comprised of a 24 hr safety study and a hemorrhagic shock-resuscitation model. In each study tissue oxygen tension, hemodynamics (including by echocardiography), and blood gas analyses were performed. Organ function markers included renal and liver function tests, creatinine kinase (marker of muscle injury), lipase (marker of pancreatic injury) and triglycerides. Cytokines (IL-6, IL-10) and markers of complement activation (C3a, C5a) were also measured.

Results and Discussion: Our engineered recombinant HBOC incorporates many unique and innovative features, designed to make our HBOC more stable and less redox reactive. This includes the incorporation of through-protein 'electron wires' that significantly impact on the ability of Hb to initiate damaging oxidative reactions. We have also used fetal Hb as the foundation of the HBOC due to its greater stability. Our HBOC combines these features with NO scavenging mutations and a novel homogeneous PEGylation system that does not affect oxygen binding cooperativity.

Our *in vitro* studies demonstrate that the engineered recombinant HBOC is significantly less reactive and more stable than earlier generations of HBOCs. The PEGylated HBOC reduced biomarkers of oxidative damage in a hemorrhage-reperfusion model. Furthermore, animals with critically low blood pressure are effectively rescued with significantly higher survival rates compared to volume expanders.

Molecular oxygen activation in the cofactor-less formyl glycine generating enzyme investigated using dose-resolved X-ray crystallography

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Formylglycine-generating enzyme (FGE) is a recently discovered enzyme that catalyses conversion of a cysteine to formyl-glycine, a critical cotranslational modification for type I sulfatase activation. Outside of its biological context, FGE has been employed as a biocatalyst for bioconjugation applications, generating significant interest in elucidating the reaction mechanism of FGE to enhance its native reactivity for biotechnology applications. FGE is a co-factor free oxygenase, enzyme that can directly activate O_2 through a combination of specific amino acid residues in its active site abstracting an electron and a proton simultaneously (proton coupled electron transfer; PCET) from a bound substrate molecule. Early research suggests that in FGE the reaction is carried out by a pair of conserved cysteine residues which form a disulfide bond in resting state. Upon reduction, one cysteine engages the substrate while the other reacts with molecular oxygen. In this study, we employed extensive dose-resolved X-ray crystallographic techniques to investigate the catalytic mechanism of FGE from *Streptomyces lividans*. Using room temperature serial femtosecond X-ray crystallography (SFX), we resolved a radiation damage-free structure of FGE in its resting state, revealing the precise oxygen-binding site for the first time (Figure 1A). Additionally, X-ray exposure was used to initiate and drive the catalytic cycle in the absence of substrate (Figure 1B and 1C), enabling the capture of a previously postulated intermediate: cysteine sulfenic acid (CSO) (Figure 1B). These findings provide critical structural insights into the FGE catalytic mechanism and advance our understanding of cofactorfree oxygenase function.

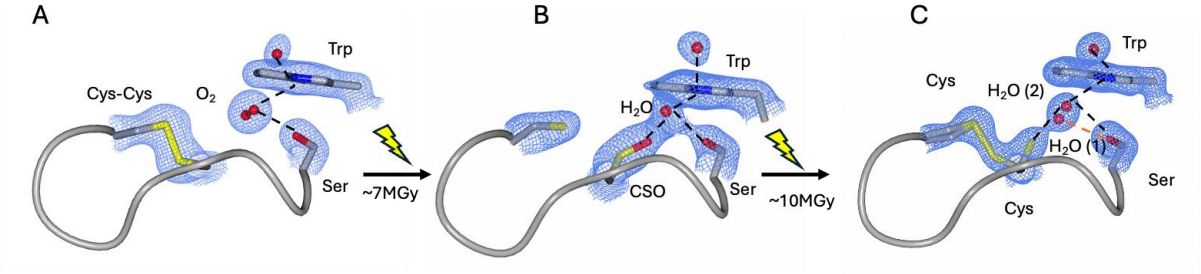

Figure 1. Selected states of the FGE active site. Black dashed lines indicate hydrogen bonds. A) Resting state of the FGE active site, determined using room temperature serial femtosecond crystallography (SFX). B) Cysteine sulfenic acid (CSO) intermediate, observed upon X-ray exposure. C) Further X-ray exposure leads to the formation of a water molecule and reformation of the disulfide bond. The water molecule and cysteine residues are partially occupied. H_2O (1) is associated with the cysteine engaged in the disulfide bond, while H_2O (2) corresponds to the reduced cysteine. The orange dashed line denotes a single hydrogen bond interaction involving water1.

Selected Oral and Poster Abstracts

Characterization of globin Y in vertebrates

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Introduction

Globin Y (GbY) is one of the eight vertebrate globins and was first detected in the clawed frog (*Xenopus tropicalis*). Later, it was also detected in the platypus (*Ornithorhynchus anatinus*), in the red-throated anole (*Anolis carolinensis*), in the Chinese softshell turtle (*Pelodiscus sinensis*), in the elephant shark (*Callorhinchus milii*), in the coelacanth (*Latimeria chalumnae*) and in the spotted gar (*Lepisosteus oculatus*). However, gene loss occurred in teleost fish, birds and higher mammals. Previous expression analyses show that GbY is expressed in all tissues examined so far (brain, kidneys, liver, spleen, gills, reproductive organs), but does not have a specific/ primary site of expression. These expression analyses were all carried out using different methods (RT-PCR, qPCR, bioinformatics) based on RNA expression. Most biochemical properties like the coordination, O_2 affinity and thus the possible function of GbY proteins are still unknown.

Methods

Our bioinformatic expression analysis was performed using public SRA datasets of different species and tissues using the Salmonquant Mapper on Galaxy. GbY from spotted gar, coelacanth, clawed frog, red-throated anole, Chinese softshell turtle and platypus were recombinantly expressed via the pET expression system in *E. coli* to determine the heme coordination photometrically. Additionally, by expressing of different GbY genes in the CHSE-214 fish cell line, we aim to perform various stress experiments to draw conclusions about the possible effects of GbY on cell viability.

Results/Discussion

GbY expression analysis shows a high variability between species. It could always be detected in nervous tissue, heart, kidney, liver and gonads. The quantification hints on a main site of expression which appears to be in the testis. An exception seems to be the coelacanth, and within the reptiles there could be a change in expression the closer they are related to birds. The coordination of GbY from the spotted gar, red-throated anole, Chinese soft-shelled turtle and platypus is hexa-coordinated, while the coordination of the coelacanth and clawed frog is penta- coordinated. Neuroglobin, globin X, androglobin and cytoglobin are hexacoordinated that represents the coordination of the early evolutionary origin. For the duplicated Cygb gene in zebrafish we know that depending on the cellular environment its coordination may change between the penta or hexa arrangement. Thus, the difference in GbY coordination may indicate the same evolutionary pathway. Hemoglobin, myoglobin and globin E are pentacoordinate, which shows an improved O2-binding configuration that evolved later. The possible function of GbY will be analyze further appling fish cell culture combined with oxidative stress like hypoxia, ROS and temperature.

Conclusion

We were able to show that GbY expression varies between the species studied and that the main site of expression is in the testis. GbY is hexa-coordinated, except in the coelacanth and clawed frog which may indicate different functions. To be able to say more about the possible functions and properties of GbY in vertebrates, we are planning oxidative stress tests in cell culture.

The Role of Globin Proteins in the Transition from Water to Land

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Introduction:
The transition from water to land during the rise of tetrapods represents a milestone in vertebrate evolution, sparking considerable scientific interest in understanding the underlying molecular mechanisms. A critical aspect of this transition involves the evolution of globin proteins, which are essential for oxygen transport and storage. Our study investigates the evolution of globin proteins and their significance in this transition, with a special emphasis on lungfish as a key species. Lungfish, regarded as the closest living relatives of tetrapods, possess lungs and the capacity to breathe air, offering invaluable insights into tetrapod evolution. Interestingly, lungfish share more genetic similarities with their land-dwelling relatives than with their fish ancestors.

Methods:
We investigated the globin repertoire of the lungfish using various methods, including bioinformatic approaches to identify different globin types. We examined different tissues to narrow down their functions by analysing expression levels and structural properties.

Results/Discussion:
Our results reveal lineage-specific duplications and losses of globin genes, indicating dynamic evolutionary events. Teleost fishes exhibit a rich diversity of globin isoforms, which may contribute to their adaptation to different (aquatic) environments. Tetrapods show significant changes in globin gene expression and protein structure compared to their aquatic ancestors. Further comparative studies using recombinant gene expression and comparing different vertebrate lineages are needed to improve our understanding of this important evolutionary event.

Conclusions:
Our study highlights the individual differences between different vertebrate species and points to the critical role of globin proteins in the transition from water to land, providing insights into vertebrate evolution.

Multi-Omics Analysis of Myoglobin Knockout Zebrafish Metabolism

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Aim
Myoglobin is the oxygen carrier and storage protein of the heart and skeletal muscle, where it is believed to be crucial for sustaining aerobic metabolism but, rigorous experimental evidence has been limited.

Methods & results
Using Oroboros high-resolution respirometry on permeabilized heart fibers from myoglobin-knockout zebrafish (*Danio rerio*), generated by CRISPR-Cas technology (Hejlesen et al., 2023), we have found that myoglobin expression does not affect mitochondrial respiration. However, transmission electron microscopy revealed changes in the density, morphology and structure of heart mitochondria without myoglobin. Measuring whole-animal respiration, we found lower standard metabolic rates and higher maximal swimming speeds in zebrafish without myoglobin, possibly due to a shift in muscle fiber types. We then used advanced metabolomic MALDI-TOF imaging, capillary electrophoresis mass spectrometry (CE-MS), and transcriptomics on wild-type and knockout zebrafish exposed to hypoxia to determine whether myoglobin expression affects the metabolic rewiring taking place in the intact heart and skeletal muscle.
Surprisingly, our preliminary data shows little impact of myoglobin loss on metabolites, even in hypoxia.

Conclusions
This data suggests that, at least in adult zebrafish, heart energy metabolism is not significantly affected by the loss of myoglobin, but it impacts heart mitochondrial density and morphology, as well as whole-animal metabolic rates and swimming speed. Further investigations are ongoing to understand the implications of these findings.

Hejlesen, R., Kjær-Sørensen, K., Fago, A., & Oxvig, C. (2023). Generation and validation of a myoglobin knockout zebrafish model. *Transgenic Research*, *32*(6), 537. https://doi.org/10.1007/S11248-023-00369-3

Targeting cytochrome *bd* oxidases from *Acinetobacter baumannii* with steroid drugs

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Cytochrome *bd* respiratory oxidases couple the oxidation of reduced quinols to the generation of a proton-motive force and the reduction of molecular oxygen. These protein complexes are unique to prokaryotes and can aid growth & survival in a variety of bacterial pathogens. *Acinetobacter baumannii* encodes two cytochrome *bd* complexes, neither of which have been studied before. As steroid drugs have recently been shown to be effective cytochrome *bd* inhibitors, it was therefore of interest to investigate their efficacy against these terminal oxidase complexes in *A. baumannii*.

The *A. baumannii cydABX1* and *cydABX2* operons were cloned and the cytochrome *bd*-1 and *bd*-2 complexes were expressed as the sole respiratory oxidases in a mutant *E. coli* strain. The *bd*-1 and *bd*2 complexes were purified using affinity chromatography and gel filtration, and incorporation of the haem cofactors was confirmed using difference spectroscopy approaches. Kinetic assays were performed via monitoring the spectrakinetic changes that accompany the oxidation of duroquinol substrate, yielding k_{cat} values for *bd*-1 and *bd*-2 of 738 s^{-1} and 435 s^{-1}, respectively. Dose-response inhibition experiments with the steroid drug quinestrol yielded IC_{50} values of 12 ± 3 µg/mL and 19 ± 9 µg/mL for purified *bd*-1 and *bd*-2, respectively. Growth experiments with wild type *A. baumannii* resulted in minimal inhibition with quinestrol, although viability assays revealed a LC_{50} of 6.1 ± 2.0 µg/mL, with maximum killing plateauing at 89 %.

Quinestrol is clearly a good inhibitor of *A. baumannii bd*-I and *bd*-2 and does exhibit bactericidal effects, although the relatively low potency towards whole cells mirrors that of other Gram-negative pathogens previously tested. *In silico* docking experiments have revealed new drug candidates to target these cytochrome *bd* complexes, and future work will focus on improving the delivery of steroid drugs across the Gram-negative outer membrane to target aerobic respiration.

Hemoglobin haem capture by staphylococcal receptor IsdB: a complex process unveiled by time-resolved X-ray solution scattering.

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During colonization, *Staphylococcus aureus* takes advantage of host hemoglobin (Hb) as its primary iron source to sustain growth and settle the infection. To access hemic iron, the bacterium exploits hemolysins to lyse red blood cells' membrane and release Hb, and employs a nine-protein machinery called Iron-regulated surface determinant system (Isd) to capture it and extract heme. The initial key interaction with Hb involves the cell-wall exposed receptors IsdB and IsdH, with the former classified as a virulence factor. IsdB is a modular protein composed of two near-iron transporter (NEAT) domains with an immunoglobulin-like folding, connected by a flexible linker. Heme retrieval is achieved through a complex series of molecular events, the main steps of which are represented by Hb interception and binding by NEAT1, followed by NEAT2-mediated heme extraction. Despite the overall process being largely characterized, the structural and kinetic details underlying the singular events of the proteinprotein interaction were missing. By applying time-resolved X-ray solution scattering in combination with rapid-mixing optical absorption and fluorescence techniques, we successfully revealed the structural and kinetic steps leading to Hb heme extraction by IsdB. A first binding on Hb beta chains promotes the dimerization of the globin tetramer, making alpha subunits available to the attach of a second hemophore molecule. Only after the formation of a 2IsdB:Hb_{dimer} complex, heme can be extracted from both Hb chains, likely assuring a fully productive interaction in vivo. These findings will possibly support the design of IsdB:Hb interaction inhibitors as potential antimicrobials against *S. aureus*.

MiRNA-induced gene regulation in the hypoxia-adapted, long-lived, and cancer resistant subterranean rodent *Spalax*

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The subterranean rodents *Nannospalax galili (Spalax)* and *Heterocephalus glaber* (naked mole rat) are evolutionarily adapted to hypoxia. This adaptation is coupled with longevity and cancer resistance. RNASeq studies have recently unveiled adaptive gene-regulatory networks of *Spalax* that allow it to cope with the environmental hazards of a hypoxic environment. MiRNAs are a class of highly conserved small RNAs that form part of many such networks, and hypoxia is known to regulate the expression of a certain subset of miRNAs termed hypoxia-induced miRNAs (hypoxamiRs). Small RNA sequencing of *Spalax* heart tissue under normoxic vs. hypoxic conditions revealed 74 differentially expressed (DE) miRNAs. Interestingly, the vast majority of known hypoxamiRs did not show significant regulation. Gene Ontology (GO) analysis of the DE miRNA targets showed a strong enrichment of GO terms associated with neuron and cardiac muscle regulation, as well as with cellular stress responses. Comparison with published naked mole rat data from corresponding tissue showed a similar pattern of enriched GO terms. In contrast, the DE miRNAs between both species overlapped by only 1, suggesting evolutionary convergence on the level of biological function rather than on the transcriptional level. Our analyses indicate that miRNAs are involved in the gene-regulatory hypoxia response of both rodent species, for instance via cardiac muscle regulation. More analyses are being conducted to understand the contribution of miRNAs to the adaptive phenotype of *Spalax*.

Neuroglobin gene regulation in the long-lived, cancer and hypoxia resistant rodent *Nannospalax galili*

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The blind mole rat *Nannospalax galili* (*Spalax*), a rat-sized subterranean rodent native to North Africa and the eastern Mediterranean region, exhibits remarkable physiological adaptations including cancer resistance, exceptional longevity of up to 20 years, and an extraordinary tolerance to hypoxia. Compared to its closest relatives, the mouse and the rat, *Spalax* is capable of surviving for several hours under severely hypoxic conditions without sustaining irreversible damage. Owing to its hypoxic underground habitat, *Spalax* also can be a valuable model organism for studying the evolution of oxygen-binding proteins and their gene regulation. Among these proteins, neuroglobin (*Ngb*)—a neuronal oxygen-binding protein—has emerged as a key candidate for investigating the molecular mechanisms underlying hypoxia tolerance.

We previously conducted a quantitative analysis of *Ngb* gene expression at both the mRNA and protein levels in brain tissues of *Spalax* and rat under normoxic and hypoxic conditions. Notably, *Ngb* expression was significantly higher in the *Spalax* brain under normoxic conditions compared to the rat, suggesting species-specific regulatory mechanisms potentially shaped by evolutionary pressures. To explore the molecular basis of this differential gene expression, we hypothesised that insertions or deletions (InDels) within cis-regulatory elements like promoters, enhancers and silencers may alter transcription factor binding sites, thereby influencing gene regulation. Using a 120-species wholegenome multiple sequence alignment as part of an *in silico* study, we identified a *Spalax*-specific 8 base pair insertion within the promoter region of *Ngb*, overlapping a predicted binding site for the transcription factor *Nuclear Factor Kappa B Subunit 1* (*Nfkb1*). Functional validation via dual-luciferase reporter assays *in vitro* revealed a regulatory impact of the *Spalax* promoter variant compared to the orthologous rat sequence, supporting the hypothesis that this insertion modulates transcriptional activity.

Our findings suggest that species-specific InDels in cis-regulatory elements, such as those in the *Ngb* promoter, may contribute to the enhanced hypoxia tolerance observed in *Spalax*. This approach is currently extended to additional candidate genes to further investigate the role of InDel mutations in the adaptation of hypoxia-tolerant species, like *Spalax*, to extreme environments.

Mapping myoglobin expression in epithelial tissues using a cross-tissue single-cell atlas

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Myoglobin (MB) is predominantly expressed in striated muscle, where it facilitates oxygen storage and intracellular transport. Beyond these classical functions, MB also contributes to the scavenging of reactive oxygen and nitrogen species and the modulation of lipid metabolism, particularly in brown adipose tissue. MB expression has also been reported in epithelial tissues and epithelial-derived tumours such as breast and prostate cancer, though its role in these contexts remains unclear.

To investigate MB expression across a broad range of epithelial states, including healthy, inflamed, and cancerous tissues, we created a cross-tissue epithelial cell atlas. This atlas was built by integrating multiple publicly available single-cell RNA sequencing (scRNA-seq) datasets representing epithelial diversity across organs such as the mammary gland, prostate, lung, and gastrointestinal tract.

The pan-epithelial atlas enabled cell type-resolved analysis of MB transcript distribution across diverse epithelial states. Our analysis revealed cell type-specific expression of MB in distinct secretory cell populations, suggesting a potential non-classical role of MB linked to metabolic or secretory functions.

Our integrative approach offers a comprehensive resource for examining patterns of tissue- and cell type-specific MB expression, providing insights into the potential contribution of MB to epithelial cell identity, differentiation, and function under both normal and pathological conditions.

Characterization of bryophyte hemoglobins provides insight into ancestral functions

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Representative species from three bryophyte lineages, mosses (*Physcomitrella patens*), hornworts (*Anthoceros punctatus*), and liverworts (*Marchantia polymorpha*), contain hemoglobin genes that are ancestral to other plant hemoglobins. Despite their evolutionary significance, the kinetic properties and ligand binding characteristics of bryophyte hemoglobins remain largely uncharacterized, with only limited data available on oxygen binding capability and no comprehensive comparison across the major bryophyte groups or to higher plants.

To fill this gap and to use early evolved plants as model systems to better understand the function of the more recently evolved non-symbiotic hemoglobin proteins, we have completed ligand binding measurements and spectral studies of bryophyte hemoglobins. These studies are the first detailed characterization of early evolved land plant hemoglobins and compare representative bryophyte hemoglobins to hemoglobins of later developed plants (rice nsHb1 and soybean Lba). This comparison gives insight into which biochemical properties of plant hemoglobins have been conserved throughout evolution and provides a platform for further *in vitro* and *in planta* work.

Our results show a combination of rate constants for binding gaseous ligands and spectral characteristics among these ancient globins that do not fit neatly with rates from class 1 or class 2 globins. Spectral characterization indicates a large fraction of hexacoordination in the bryophyte globins however, ligand binding studies reveal that these proteins have association and dissociation rate constants for oxygen that are more similar to the class 2 non-symbiotic hemoglobins and the leghemoglobins. Autooxidation and rates of reduction of nitrite have also been compared to other globin proteins and have been observed to have significantly faster rates.

Results of our experiments support the phylogenetic studies that place the bryophyte family of globins at the point of divergence between oxygen transport and non-oxygen transport in the evolution of plant hemoglobin proteins. The information from these studies will inform *in planta* work, especially in *Anthoceros* which contains a single hemoglobin in its translated proteome, in clarifying the physiological roles of plant hemoglobins.

Heme Modulation of p53 and p63: Structural and Functional Insights

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Heme is a vital component of many proteins involved in biochemical processes, including electron transfer, oxygen transport and storage, and enzyme catalysis. More recently, it has also emerged as an important molecule in cellular signaling. Some hemoproteins use heme as a binding site for small diatomic gases such as oxygen, nitric oxide, and carbon monoxide. Binding of these gases can induce conformational changes that modulate protein function and activate downstream signaling pathways. In addition, heme itself can act as a signaling molecule by directly binding to proteins, altering their conformation and functional activity (Shimizu et al., 2019, *Chem. Soc. Rev.* 48:5624–5647). This direct signaling role of heme has been observed in the tumor suppressor protein p53, a central regulator of the cellular stress response (Shen et al., 2014, *Cell Rep.* 7(1):180–193). p53 is a transcription factor that maintains genomic integrity and prevents tumor development by responding to various stress signals, including DNA damage, hypoxia, oxidative stress, and more. Upon activation, p53 binds to DNA and regulates a broad network of target genes involved in apoptosis, cell cycle arrest, DNA repair, senescence, and metabolism. The loss or inactivation of p53 is one of the most common features of human cancers (Liu et al., 2024, *Cancer Cell* 42(6):946–967).

Building on previous findings that identified p53 as a heme-binding protein, our study focused on elucidating the structural impact of this interaction. Using hydrogen-deuterium exchange mass spectrometry, we examined full-length p53 and found that the DNA-binding domain undergoes the most substantial conformational changes in the presence of heme. These structural changes may underlie the observed functional suppression of p53 upon interaction with heme (Vávra et al., 2023, *J. Inorg. Biochem.* 243:112180). Extending our analysis to p63, another member of the p53 family, we found that full-length p63 also directly interacts with heme, likely through a conserved binding region present in all p53 family proteins. In both p53 and p63, heme binding was associated with destabilization and aggregation of their isolated DNA-binding domains, suggesting a shared mechanism heme-induced misfolding. These insights provide a link between heme signaling and the structural regulation of p53 family proteins, with broader implications for understanding cancer progression and inventing new therapeutic approaches targeting heme-protein interactions.

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Drug repurposing to target the *Mycobacterial* respiratory oxidase cytochrome *bd*

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Tuberculosis is one of the most widespread and deadliest diseases currently, with a quarter of the global population estimated to be infected with *Mycobacterium tuberculosis* (MTb), and 5-10% developing symptoms[1]. Antimicrobial resistance is a significant problem with multidrug-resistant MTb increasing in prevalence by approximately 10% a year, new anti-mycobacterial drugs are desperately needed[1]. Mycobacteria rely upon aerobic respiration for proliferation during infection, and there has been much recent interest in targeting mycobacterial respiratory complexes with novel drugs, including the cytochrome *bd* complex that is only found in prokaryotes. Herein, we investigate the efficacy of a known steroid drug inhibitor of cytochrome *bd* upon *M. smegmatis* and employ a combination of *in silico* and laboratory techniques to identify potential anti-mycobacterial compounds.

A respiratory mutant strain of *Mycobacterium smegmatis*[2] was used to generate '*bd*-only' membranes that contain cytochrome *bd* as the sole respiratory oxidase, and complex assembly was confirmed using difference spectroscopy approaches. Dose response assays were performed using a new assay that monitors spectrakinetic changes during duroquinol oxidation, which revealed an IC_{50} of 14.4 ± 1.5 µg/mL for the steroid drug quinestrol.

Subsequent work demonstrated for the first time the successful purification of *M. smegmatis* cytochrome *bd* that had been recombinantly expressed in *E. coli*, and kinetic analyses yielded a k_{cat} of 7453 s^{-1}. Dose-response inhibition experiments with the purified *M. smegmatis* cytochrome *bd* and quinestrol yielded an IC_{50} value of 6.7 ± 4.3 µg/mL. Growth and viability experiments with wild type *M. smegmatis* cells revealed minimal activity for quinestrol, indicating that the mycobacterial cell envelope is a significant barrier to steroid drugs.

Computational screening of drug libraries revealed additional potential inhibitors of mycobacterial cytochrome *bd* and the top-ranking compounds have been assessed using kinetic approaches, paving the way for the identification of novel anti-mycobacterial agents.

[1]WHO, Global tuberculosis report, 2024
[2]Matsoso, L. G. *et al. J. Bacteriol.* 187, 6300–6308 (2005)

Cytoglobin: A potential respiratory regulator in retinal cells

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Oxygenation of the retina is challenged by the need for an unobstructed light path to the photoreceptors because oxygen-carrying hemoglobin in the blood absorbs photons. Cytoglobin has the potential to facilitate retinal oxygen diffusion or prevent oxidative damage in this oxygen-sensitive tissue. However, the function of cytoglobin in the retina remains unknown. In this study, we analyzed single-cell RNA sequencing data from mouse retinas for co-expression patterns to elucidate the role of cytoglobin. We show that the highest cytoglobin levels are found in the interneurons of the inner nuclear layer, particularly the GABAergic amacrine cells. By testing the genes co-expressed with cytoglobin for enriched gene ontologies, we inferred patterns of functional relationships with cytoglobin. Our results strongly support a tight coupling of cytoglobin to the energy state, particularly to the NADH dehydrogenase complex of the electron transport chain. Based on the overrepresentation of ubiquitinrelated proteins, we propose a mechanism of respiratory regulation in which oxygen sensing by cytoglobin regulates the NADH dehydrogenase complex through ubiquitination, serving as a signal for protein degradation by the proteasome. Our study provides new insight into the role of cytoglobin in the retina as a potential oxygen-sensitive regulator of respiration, and it broadens the perspective of possible molecular associations and functions of globin proteins.

Resurrecting lost ancient myoglobins to elucidate mechanisms of diving adaptation in mammals

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Introduction

The combination of modern data science and bioengineering techniques enables the resurrection of extinct proteins using statistically deduced ancestral sequences. This approach, known as “molecular archeology,” provides insights into biological traits given via evolution. We applied this method to investigate the evolutionary adaptations of myoglobin (Mb), which enables deep diving in mammals [1,2].

Methods

Ancestral Mb sequences of whales and seals were inferred from molecular phylogenies and synthesized. The resulting proteins were experimentally and theoretically characterized by X-ray crystallography, small-angle X-ray scattering (SAXS), solubility assays using polyethylene glycol (PEG), denaturation assays, molecular dynamics simulations, and solvation energy calculations. We collected parameters such as the surface charge (Z_{Mb}), second virial coefficient (A_2), and precipitation resistance (β) and conducted principal component analysis (PCA) to identify dominant adaptive features.

Results and Discussion

During the early phase of evolution, Z_{Mb} and β increased during aquatic re-adaptation in both whales and seals, enhancing electrostatic repulsion and preventing aggregation at high concentrations. While solubility ($\log S_0$) did not consistently increase, β was strongly correlated with Z_{Mb} [3,4]. Late phase evolution improved thermodynamic stability (ΔG_{fold}), suggesting a two-phase adaptation model. PCA revealed that β and intramolecular interactions were key adaptive traits, whereas solubility ($\log S_0$) played a lesser role.

Conclusions

The increased precipitation resistance via electrostatic tuning and improved thermodynamic stability highlight a robust molecular strategy for diving capability. This approach not only enriches our understanding of protein evolution but also provides strategies to improve the solubility and stability of therapeutic proteins.

[1] Isogai, et al. (2018) Sci. Rep. 8, 16883. [2] Isogai, Y. et al. (2021) iScience 24, 102920. [3] Isogai, et al. (2022) Biochemistry 61, 1543-1547. [4] Imamura et al. submitted.

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The multiplicity of the *Caenorhabditis elegans* globin family.

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Over the last few decades, the model organism *Caenorhabditis elegans* has been crucial for a deeper understanding of biological processes at the genetic and molecular level. The number of protein coding genes in its genome is comparable to that of humans and some of its gene families underwent lineage-specific expansion, such as G-protein coupled receptors (GPCRs), neuropeptides, insulins, and globins. The *C. elegans* genome encodes 34 globins, of which many are predicted to be alternatively spliced, leading to an impressive set of 55 different globin proteins. Most of these have not been functionally characterized, despite intense research activity in the *C. elegans* community worldwide. This raises the question how this 1-mm worm, consisting of only 959 somatic cells, employs such a variety of globins.

We have exploited the abundance of *C. elegans*-based omics and expression data and used bioinformatic prediction tools to explore the potential functional diversity of the *C. elegans* globin family. Integrated whole-organism proteomics data shows that almost all *C. elegans* globins appear in low quantities (0-7 ppm) indicating that most globins function very locally or require low protein abundance for their functionality (such as signaling). With a very high protein level of 462 ppm, GLB-1 is an exception and may therefore have a bulk function, such as oxygen storage or transport.

Reporter expression analysis and single cell RNAseq reveal that the majority of globins are predominantly expressed in specific but overlapping sets of neurons. These sets may be functionally related, like motor- or sensory neurons, or may cover a broad range of functionally unrelated neurons. More specifically, some globins are almost exclusively expressed in a single neuron (e.g. GLB-31 in the RIA neuron) while others have a broad expression pattern (e.g. GLB-18 in virtually all neurons). While most globins are restricted to the neuronal system of the worm, some exhibit additional expression in other tissues such as glia, hypodermis, muscles, somatic gonad and intestine. The mode of action of globins seems to be complex, as through different life stages the location as well as the level of expression varies.

This variation among *C. elegans* globins seem to appear at the intracellular level as well: at least 15 of the 55 isoforms are predicted to be targeted to the mitochondria, one may be linked to the endoplasmic reticulum and 14 are predicted to be lipidated, possibly for membrane association. GLB-33 is attached to the membrane by its GPCR domain. This membrane anchoring of globins suggests a function in local (redox) signaling.

Except for GLB-1, the *C. elegans* globins contain variable N- and/or C-terminal extensions of 8 - 372 amino acids that, besides some targeting sequences, do not reveal recognizable domains. Neither are these extensions studied extensively on their potential function. The few exceptions we can highlight are the long N-terminal 372-amino acid extension of GLB-33, harbouring a GPCR domain and the essential extensions of GLB-3 for increased protein stability at low pH. Besides membrane anchoring and protein stability, these extensions may be vital for protein-protein interactions.

The wide divergence in expression patterns, intracellular localization, and terminal extensions observed in *C. elegans globins*, may indicate that the complexity of its globin family compensates for its anatomical simplicity and therefore allows this animal to thrive in a complex and strongly fluctuating soil environment

The role of GLB-1 in stress resistance and metabolism in *Caenorhabditis elegans*

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The genome of the nematode model organism *Caenorhabditis elegans* encodes 34 globin genes, exhibiting remarkable structural and sequence diversity. Most globins are predominantly expressed in low amounts in neurons, although a subset localizes to additional tissues, including glia, muscle, and the reproductive and excretory systems. Among these, globin 1 (GLB-1) is atypical due to its high abundance (top 5% of all proteins), its small size, the lack of both N- and C-terminal extensions, and its expression across multiple tissues. The structure of this myoglobin-like protein is well-characterized and known to reversibly bind oxygen with high affinity. Its transcriptional regulation is governed by the Forkhead box O transcription factor DAF-16 and the hypoxia-inducible factor HIF-1, leading to strong upregulation under various stress conditions, including hypoxia (≤1% O_2), while remaining unchanged during anoxia (0% O_2).

In this study, we investigated the mode of action of GLB-1 in *C. elegans* under normoxia from different perspectives. We characterized the expression pattern of *glb-1* and found that *glb-1* is primarily expressed in the head (head muscles, glia, and/or arcade cells) and vulval muscles. Furthermore, it is present in the oxygen-sensing AQR, PQR, and URX neurons, as evidenced by co-expression with the guanylate cyclase GCY-32 marker. *glb-1* has been identified earlier as one of the most upregulated genes in *daf-2* mutants, which we here confirm. In *daf-2* knockdowns, which have a drastically extended lifespan and increased stress resistance due to constitutive activation of DAF-16, we show that *glb-1* is particularly induced in the hypodermal seam cells. This local induction specifically depends on DAF-16. Although these findings suggest that GLB-1 may be involved in worm longevity and stress resistance, our results indicate otherwise: we found no significant contribution of GLB-1 to longevity or resistance to heat and tert-butyl hydroperoxide (TBHP)-induced oxidative stress. However, in the *daf-2* mutants it plays a minor role in hydrogen peroxide resistance. Despite its oxygen-binding properties, GLB-1 does not affect metabolic rate under normoxic conditions but appears to modestly reduce ATP levels in both wild-type and *daf-2* mutant worms. This ATP reduction correlates with altered fat content, as *glb-1* mutants exhibit a slightly leaner phenotype compared to controls.

Overall, our findings demonstrate that, under normoxia, *glb-1* is constitutively expressed in head tissues and a subset of neurons, including oxygen-sensing neurons, while DAF-16 activation can induce its expression in seam cells. While GLB-1 has minimal impact on heat and oxidative stress resistance—except for a slight effect on hydrogen peroxide tolerance—it likely influences metabolism by decreasing systemic ATP levels and fat stores. Future research will focus on the function of GLB-1 under hypoxic conditions.

Targeting cytochrome *bd* oxidases from *Klebsiella pneumoniae* with steroid drugs

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The continued emergence of antimicrobial resistance is a burgeoning global health issue, demanding urgent attention and the need to drive for novel antimicrobial development. We propose a development route through the utilisation of current FDA-approved drug libraries to re-purpose, re-develop, and refine drugs to current antimicrobial targets of interest. We focus on one such promising drug target named cytochrome *bd* oxidases; they are respiratory terminal oxidases solely found within prokaryotes which aid in the generation of the proton motive force through coupling the oxidation of reduced quinols to the generation of a proton-motive force and the reduction of molecular oxygen. Preliminary work in the Shepherd lab has identified that steroid drugs inhibit cytochrome *bd* in both *Escherichia coli* and *Staphylococcus aureus* (Henry *et al.* 2024). Herein, we aim to further determine the capacity of these compounds to inhibit cytochrome *bd* complexes from another Gram-negative bacterial pathogen, *Klebsiella pneumoniae*.

The *K. pneumoniae cydABX2* operon was cloned and the cytochrome *bd*-2 complex was expressed as the sole respiratory oxidase in a mutant *E. coli* strain. The *bd*-2 complexes were purified using affinity chromatography and gel filtration, and incorporation of the haem cofactors was confirmed using difference spectroscopy approaches. Kinetic assays were performed via monitoring the spectrakinetic changes that accompany the oxidation of duroquinol substrate, yielding k_{cat} values for *bd*-2 of 50.5 s^{-1}. Dose-response inhibition experiments with the steroid drug quinestrol yielded an IC_{50} value of 1.31 µg/mL +/- 0.47 µg/mL for purified *bd*-2. Growth experiments with wild type *K. pneumoniae* resulted in minimal inhibition with quinestrol, although viability assays revealed a LC_{50} of 6.020 +/- 2.744 µg/mL, although maximum killing plateaued at only 12%.

Our findings demonstrate that quinestrol is a good inhibitor of purified cytochrome *bd* complexes from a range of bacterial pathogens although potency against Gram-negatives appears to be limited. Hence, future work on steroids will focus on derivatisation to enhance drug delivery to the inner membrane of a variety of Gram-negative pathogens.

References

Henry, S. A. *et al.* (2024) J. Infect. Dis. **230**, e149–e158

The enhanced hemoglobin function can be explained by additional tertiary structural changes exerted by changes in hydrophobicity in the α1β1 interface

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It is widely accepted that the classical Two-State allosteric model, namely, the Monod-Wyman-Changeux (MWC) model, adequately explains the functional behavior of tetrameric hemoglobin (Hb), *i.e.*, changes in affinity for oxygen are due to a transition between two quaternary states: T (low oxygen affinity), and R (high oxygen affinity). After measuring oxygen binding isotherm over a wide range of solution conditions and in the presence of heterotropic effectors, we found that allosteric constants for T and R (namely, K_T and K_R, respectively) are not constant, since they vary in large extent (about 2,000 times in the case of K_R).

Inspection of available structural data revealed that changes in oxygen affinity correlated with tertiary changes in the heme pocket caused by additional interactions in the central cavity provided by specific allosteric effectors. For instance, we concluded that the docking of bezafibrate or the compound L35 in the α1β1 interface, contributed with additional hydrophobic interactions in the central cavity, and were responsible for such a dramatic modulation of oxygen affinity in Hb. To test this idea, we prepared chemically-modified Hbs in specific cysteine residues (Cys104α, Cys112β), which are located in the α1β1 interface, using two types of dithiopyridine reagents, namely, 2,2'- and 4,4'-Dithiopyridine.

We found that Hb samples modified with 2,2'-Dithiopyridine in the β-subunit (Cys112β) exhibited a marked decrease in the affinity for oxygen. This was dramatically enhanced upon the additional modification of the residue in the α-subunit (Cys104α). In this case, the transition from T low-affinity to R high-affinity state of this doubly-modified Hb was completely hindered, *i.e.*, the modified Hb remained in an extremely low-affinity T-state even upon complete oxygenation. In contrast, the Hb modification of only Cys104α with 2,2'-Dithiopyridine produced a modified Hb that exhibited a somewhat increased affinity of the T-state. In contrast, modification of both cysteine residues with 4,4'-Dithiopyridine produced a Hb with a somewhat decreased affinity in both the T- and R-states.

We concluded that those differences in polarity between the modifying moieties of 2- and 4-Thiopyridyl facing the central cavity were responsible for the dramatic functional changes observed. In other words, an increase in hydrophobicity in the central cavity results in a Hb with an extremely decrease affinity for oxygen. These results cannot be explained by simply invoking the classical two-state (T-R) allosteric model.

High-throughput assay for quinol-binding respiratory complexes

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Cytochrome *bd* respiratory oxidases couple the oxidation of reduced quinols to the generation of a proton-motive force and the reduction of molecular oxygen. These protein complexes are unique to prokaryotes and can aid growth & survival in a variety of bacterial pathogens. Conventional approaches to assay respiratory oxidases have relied upon measuring oxygen consumption using an oxygen electrode, which provides high quality data but is low throughput. Oxygen-sensitive fluorophores can be used in a plate reader, although these can be prohibitively expensive for larger screening experiments. To this end, a new assay was developed via monitoring duroquinol oxidation at 260 nm via spectrakinetics in a UV-vis spectrophotometer and single wavelength measurements in a plate reader. This approach was used to investigate inhibitor binding to *E. coli* cytochrome *bd*-I.

To validate the new high-throughput assay we compare cytochrome *bd*-I kinetic measurements to data obtained using oxygen electrodes and fluorimetric approaches. Furthermore, previous work in the Shepherd lab has reported steroid drugs as potent inhibitors of cytochrome *E. coli* cytochrome *bd*-I (Henry *et al.* 2024), although the modes of binding have not previously been investigated using laboratory approaches. Hence, we have employed our high-throughput assay to determine apparent K_m values for duroquinol, and have conducted inhibition kinetics to investigate the binding location for the steroid drug quinestrol. The methodological advances herein will be of interest to those working on any quinol-oxidising respiratory complex, and we intend to exploit this approach for broader drug screening of cytochrome *bd* complexes, a topic of much interest over the past decade.

References

Henry, S. A. *et al.* (2024) J. Infect. Dis. **230**, e149–e158

Biomicking *Staphylococcus aureus* IsdB hemophore for the development of hemoglobin-binding peptides

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Hemoglobin (Hb) is densely packed within erythrocytes to exert oxygen delivery. However, during hemolysis – a pathological condition involving the rupture of erythrocytes – Hb is released into the plasma. Hemolysis can be triggered by drugs, toxins, genetic disorders, blood transfusions, or extracorporeal circulation procedures. The presence of free Hb in plasma can have several harmful effects, such as vasoconstriction or kidney injury, and also poses analytical challenges for blood testing, as it interferes with many routine clinical biochemical assays. Consequently, free Hb scavengers may represent a promising strategy to address both medical and analytical issues.
In this study, we drew inspiration from IsdB, a hemoglobin-binding protein (hemophore) produced by *Staphylococcus aureus* to scavenge heme and acquire iron, using it as a biomimetic scaffold. The elucidation of the structure of IsdB-Hb complex by cryo-EM (1) and solution X-ray scattering techniques (2) provided the basis for designing a peptide library. These sequences aimed to mimic an unstructured loop of IsdB which folds into a helix when the hemophore binds Hb. The designed peptides were optimized to promote loop helicization in order to bind Hb without the entire protein scaffold.
Peptides were synthesized using microwave-assisted solid-phase synthesis and subsequently characterized for their secondary structure and Hb-binding affinity using different biophysical techniques. This study led to the identification of a peptide constrained by a synthetic brace (stapled peptide) that binds Hb with a micromolar dissociation constant, suggesting that proper positioning of the stapling favors helix formation and binding to Hb.

References

(1) De Bei, O. et al. Cryo-EM structures of staphylococcal IsdB bound to human hemoglobin reveal the process of heme extraction. Proc Natl Acad Sci USA. 2022 Apr 5;119(14):e2116708119.
(2) De Bei, O. et al. Time-resolved X-ray solution scattering unveils the events leading to hemoglobin heme capture by staphylococcal IsdB. Nat Commun. 2025 Feb 17;16(1):1361.

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Can hemoglobin variants affect *Staphylococcus aureus* ability to steal host iron?

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Staphylococcus aureus is a commensal microorganism that colonizes about one-third of the human population. Due to its disposition to cause infections, ranging from mild skin infections to fatal systemic diseases, it has been prioritized by the World Health Organization for the search for new antimicrobials, with particular attention given to methicillin-resistant strains (MRSA).
S. aureus sustains its growth during infection by relying on the availability of host iron, which is stored bound to proteins either directly or as a heme cofactor. In humans, hemoglobin (Hb) binds about 60% of the body's iron. *S. aureus* exploits the iron surface determinant (Isd) system, a protein machinery that binds Hb, extracts and internalizes hemic iron into bacterial cell. The cell wall anchored-receptor IsdB is the initial component of this system, and must establish a stable and extensive network of contacts for a productive interaction with Hb. It is known that IsdB binds human Hb more efficiently than mouse or primate Hbs, and this species-specific interaction may result from co-evolutionary pressure between host and pathogen. Analogously, some human Hb variants (Hbvs) such as HbS in malaria, were evolutionarily selected for their protective behavior against infections.
To date, several hundred Hbvs have been documented, and their true prevalence is likely underestimated because many of them are asymptomatic and were discovered incidentally. Building on available structural data of the IsdB:Hb complex, we have identified on Hb the key residues involved in the protein-protein interaction, aiming at the individuation of potential polymorphisms that interfere with iron acquisition by *S. aureus*. These Hbvs are recombinantly expressed in an *Escherichia coli* host, and their ability to serve as substrates for IsdB is characterized *in vitro* and compared to the wild-type protein. Selected Hbvs with mutations able to perturb IsdB activity are tested in microbiological cultures to evaluate *S. aureus'* ability to exploit them as the sole iron source in iron-restricted conditions.
The findings will enhance our understanding of how Hb variants influence *S. aureus* iron acquisition, offering new perspectives on *S. aureus*-human interactions and shedding light on individual differences in susceptibility to infection.

CRISPR/Cas9-mediated knockouts of globins 1, 2 and 3 in Drosophila melanogaster

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Globins are phylogenetically ancient proteins and thus also belong to the standard gene repertoire of arthropods. Drosophila melanogaster contains three globins: globin 1 and two other, rather diverged gene duplicates, globins 2 and 3. Despite an early discovery in 1999 and 2006, the biological function of all 3 globins remains largely unclear. While globin 1 is expressed at different levels in the fat body and tracheole cells across all developmental stages, globin 2 and 3 are only expressed in testes tissue. Expression of globins 2 and 3 begins in the late third instar larval stage, with the highest levels measured in male adult flies.

Three different globin 1 RNAi knockdown systems have been generated and suggested a diversity of glob 1 deficiency phenotypes, ranging from a reduction in lifespan under hypoxia and normoxia to severe developmental defects. Knockdown of globins 2 and 3 suggested a ROS-protective role in spermatogenesis which was further supported by unpublished experiments, revealing defects in spermatogenesis in globin 2/3-deficient strains. However, residual expression and potential off-target effects complicated the interpretation of these RNAi results. We therefore established genetic knockout systems for all three globin genes using the CRISPR/Cas technique.

Phenotypic analyses of globin 1 KOs so far did not reveal any developmental defects or evidence for a reduced life span under normoxic conditions. Under experimental hypoxia conditions, however, a reduction in lifespan was observed. Furthermore, KO flies showed a prolonged recovery timed after anoxia exposure compared to wild type, and a reduction in lifespan under oxidative stress. Hypothesizing a globin role in lipid metabolism, fat droplet volume within the fat body was studied, but did not reveal differences between KO and wild type. However, an extension of lifespan under starvation was observed, demonstrating a possible link between fat metabolism and globin expression. In the case of globins 2 and 3, our preliminary studies so far -in contrast to previous RNAi experiments- failed to demonstrate a reduction in fecundity/fertility or defects during spermatogenesis. Currently, we compare the transcriptomes of KO and wild-type flies to infer possible subtle molecular phenotypes. Additionally, further phenotypic analysis will involve different stress regimes to find the function of the Drosophila melanogaster globins. The so-far obtained differences in the results from knockdown and knockout experiments demonstrate the need for true genetic KO events for functional analysis of phenotypical defects.

Guaiacol mitigates H_2O_2-Induced Heme Degradation in Human Neuroglobin

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Neuroglobin (Ngb) is a 151-amino acid monomeric globin discovered in 2000 in human neurons [1]. Its structure resembles that of myoglobin (Mb) and hemoglobin (Hb) subunits, featuring 8 α-helices embedding a bis-histidyl six-coordinated heme *b* [2]. Ngb presents an intramolecular disulphide bridge in oxidizing condition between Cys46 and Cys55, which weakens the bond between the distal His64 and the iron causing an increase of affinity towards exogenous ligands [3]. Primarily expressed in retinal cells and neurons, Ngb plays a role in cell survival under hypoxic/ischemic or oxidative stress conditions and in neurodegenerative diseases [4]. Ngb shows different pseudo-enzymatic properties towards different potentially physiological relevant substrates [5].
Previous analysis of the reaction between Ngb and H_2O_2, a reactive oxygen species (ROS) present in the cell under physiological and oxidative conditions, showed that the protein apparently doesn't form the ferryl (oxoFe(IV)) species unlike metMb and metHb [6]. More recently, it was demonstrated that Ngb undergoes an H_2O_2-induced heme degradation which is similar but remarkably slower compared to other globins (in the timescale of hours against minutes), suggesting that the formation of the oxoFe(IV) species occurs although it's probably hampered due to the largely predominant bis-His ligated heme, acting as a barrier for the formation of the initial hydroperoxo derivative [7].
To further investigate the possible involvement of the ferryl species in the presence of H_2O_2, we have analysed the reactivity of WT human Ngb and its C46AC55A mutant, unable to form the disulphide bridge, in presence of an easily oxidizable substrate, Guaiacol (2-metoxy-phenol), which is commonly used to detect ferryl group formation [8].
The kinetics of H_2O_2-induced heme degradation, under different substrate and H_2O_2 concentration at 37°C, was studied by analysing the time-dependent evolution of the Soret band of human Ngb and of tetraguaiacol formation through UV-Vis electronic absorption spectroscopy
In the presence of guaiacol, a significant slowdown of degradative oxidation of the heme is observed (around 30-fold for WT and 20-fold for the C46AC55A mutant). Moreover, high guaiacol concentrations seem to inhibit the reaction, suggesting a possible allosteric control by interaction with Ngb. This finding indirectly supports the formation of ferryl species in human Ngb and their involvement in oxidative degradation, which is hampered by guaiacol thanks to its fast reaction with oxidized heme species.

References:

1. Burmester, Weich, Reinhardt, Hankeln, *Nature*, **2000**, *407*, 520-523.
2. Pesce, Dewilde, Nardini, Moens, Ascenzi, Hankeln, Burmester, Bolognesi, *Structure*, **2003**, *11*, 1087-1095.
3. Hamdane, Kiger, Dewilde, Green, Pesce, Uzan, Burmester, Hankeln, Bolognesi, Moens, Marden, *J. Biol. Chem.*, **2003**, *278 (51)*, 51713-51721.
4. Ascenzi, Di Masi, Leboffe, Fiocchetti, Nuzzo, Brunori, Marino, Mol. Aspects Med., **2016**, *52*,1-48.
5. De Simone, Sbardella, Oddone, Pesce, Coletta, Ascenzi, *Cells* **2021**, *10*, 3366.
6. Herold, Fago, Weber, Dewilde, Moens, *J. Biol. Chem.*, **2004**, *279 (22)*, 22841-22847.
7. Di Rocco, Bernini, Battistuzzi, Ranieri, Bortolotti, Borsari, Sola, *FEBS J.*, **2023**, *290*, 148-161.
8. De Oliveira, Santos, Buffon, *Food Res Int*, **2021**, *143*, 110266.

Site-selective PEGylation of purified hemoglobin: by linchpin-directed modification

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In the field of Hemoglobin Based Oxygen Carriers (HBOCs), PEGylation is one of the most adopted approaches to increase the hemoglobin hydrodynamic radius, to reduce side effects such as renal filtration, extravasation and increase protein stability. This protein chemical modification, extensively used in protein therapeutics, offers favorable pharmacokinetic benefits, with notable examples including Hemospan and Euro-PEG-Hb that exhibit a longer lifetime and no extravasation. However, although these products have reached the preclinical or clinical trial stages, they have encountered serious obstacles mainly associated with the occurrence of adverse effects, which have resulted in products failing clinical approvals. Structural investigations of PEGylated hemoglobin have identified two causes that may contribute to the observed adverse effects. The first concerns the heterogeneity in the degree of PEGylation, which refers to the variability in the number of PEG molecules bound to hemoglobin, responsible for conjugating instability and possible structural and functional alterations. The second is related to the increased dissociation of the tetramer after PEGylation which causes a decrease in molecular size, structural and functional heterogeneity.

We approach hemoglobin PEGylation using a recently proposed site-selective protein chemical modification, known as linchpin-directed modification (LDM). This chemistry exploits bifunctional molecules to achieve a direct, homogeneous and irreversible modification of protein on histidine residues with a biorthogonal group suitable for PEG anchoring. Considering the well-established use of PEG for the decoration of therapeutic proteins and the limitations of current PEG-based HBOCs, we employed LDM as a novel and precise anchoring method for hemoglobin PEGylation.

A structure-based computational approach was employed to select molecules that avoid targeting hemoglobin's functional sites - such as the proximal histidine – ensuring homogeneous protein PEGylation. As a result, we obtained a homogeneously PEGylated hemoglobin that, despite showing a loss of cooperativity and an increase in oxygen affinity - probably due to a non-specific effect of PEG moiety on Hb functionality - preserves its native tetrameric structure without an increased dimerization.

The *bd*-type Cyanide Insensitive Oxidase from the multidrug-resistant pathogen *Pseudomonas aeruginosa*: interaction with gaseous ligands

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Carbon monoxide (CO), nitric oxide, and hydrogen sulfide are well-known toxic gases that bind tightly and inhibit haem-containing proteins, including respiratory terminal oxidases. These three gaseous molecules act as endogenous signalling molecules at low concentrations and exhibit antimicrobial properties. This last feature is used by the host to fight infections. Some bacteria, such as the multidrug-resistant (MDR) pathogens *Escherichia coli* and *Mycobacterium* (*M.*) *tuberculosis*, defend themselves against the host immune response by overexpressing *bd*-type oxidases, which are copper-free terminal oxidases resistant to various stressors [1]. The respiratory chain of *Pseudomonas aeruginosa* (*Pa*), one of the most critical MDR pathogens, comprises four haem-copper terminal oxidases and a *bd*-type one, the Cyanide Insensitive Oxidase (CIO), which protects *Pa* against sulfide and nitrosative stress [2]. To better understand the role of CIO during *Pa* pathogenesis, we investigated its sensitivity to CO. For this purpose, we recombinantly expressed, purified and biochemically characterised CIO, which shows no spectroscopic heme *d* features. Interestingly, in contrast to the behaviour observed with *M. smegmatis bd* oxidase [3], oxygraphic measurements prove that CO potently inhibits CIO activity in competition with oxygen. These data reveal a different response to CO among *bd*-type oxidases that may arise from variations in the structural organisation of active sites and their specific environments [4] and suggest that host-produced or exogenously supplied CO may exert a significant antibacterial effect on this pathogen when CIO is expressed, opening up possibilities for CO use as a therapeutic agent for *Pa* infections.

1. Borisov VB, Giardina, G, Pistoia G, Forte E Cytochrome *bd*-type oxidases and environmental stressors in microbial physiology, *Advances in Microbial Physiology*, 86, **2025**,199-255

2. Nastasi R, Caruso L, Giordano F, Mellini, M, Rampioni G, Giuffrè A, Forte E. Cyanide Insensitive Oxidase confers hydrogen sulfide and nitric oxide tolerance to *Pseudomonas aeruginosa* aerobic respiration. *Antioxidants* **2024**, *13*, 383 3. Bayly K, Cordero, PRF, Kropp, A, Huang, C, Schittenhelm RB, Grinter, R, Greening C. Mycobacteria tolerate carbon monoxide by remodeling their respiratory chain. *mSystems* **2021**, 6, e01292-01220

4. Borisov VB, Forte E. Carbon monoxide and prokaryotic energy metabolism. *Int. J. Mol. Sci.* **2025**, 26, 2809

Neuroglobin and Cytoglobin – Investigation of Functional role in the retina.

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Introduction: Neuroglobin (Ngb) and Cytoglobin (Cygb) are relatively recently discovered proteins with intriguing distribution in the retina and in the brain. These proteins have characteristics that are similar to those of other well described members of the globin family, Myoglobin and Haemoglobin. Ngb and Cygb are thought to be involved in oxygen binding, oxidative stress and cell protection. It seems logical that these two proteins are present in the extreme oxygen environment of the retina and the brain for essential purposes, however these functions are yet to be identified. Using mouse (knockout and normal) and human retinal tissue we examined the distribution of Ngb and Cygb by specific cell type (structure) and review the Oxygen biology in layers of the retina, in order to help ascertain their role.

Methods: We used IBEX Multiplex immunofluorescent imaging (https://www.biorxiv.org/content/10.1101/2024.02.28.582563v2) on Ngb and Cygb Knockout vs control (normal) tissue to identify protein distribution in specific cells: Ganglion, nerve fibre, vasculature, horizontal, amacrine, bipolar cells, rods, cones and nuclei and correlate Ngb and Cygb protein distribution using specific antibodies which are validated through testing involving knock-out models. We examine these findings in the context of data on retinal oxygen levels.

Results:

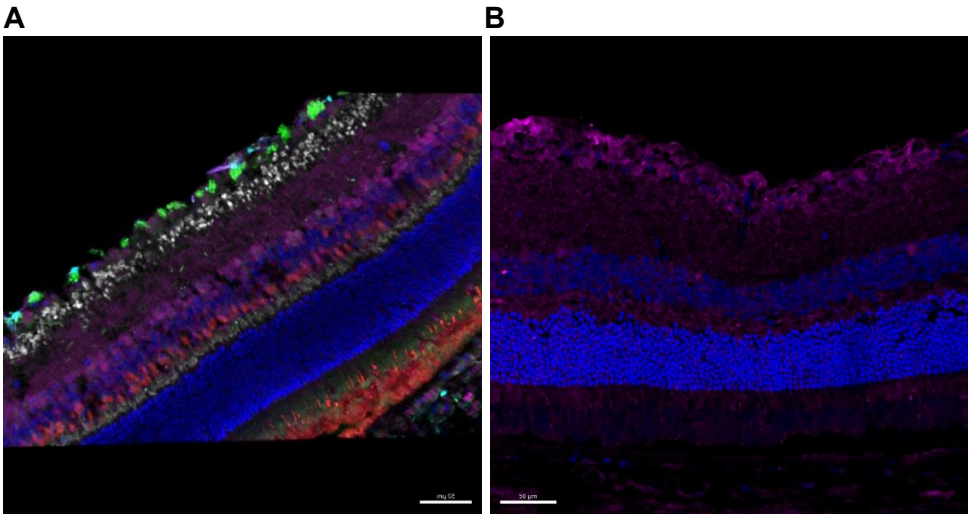

Figure 1: A Cytoglobin Mouse retina section with Cytoglobin staining (purple) Colocalised with specific cell types. Cytoglobin is widely distributed in multiple cell types (ganglion cells, horizontal cells, Amacrine cells, bipolar cells, nerve fibres/ dendrites. Neuroglobin in localised to Melanopsin cells. **B** Neuroglobin expression is limited to some retinal ganglion cells.

Discussion: Cytoglobin is found in a wide range of cell types and some of these cells have a very high level of oxygen metabolism. Neuroglobin is mostly found in intrinsically photosensitive retinal ganglion cells. We will discuss Oxygen metabolism in the retina.

Conclusions: Neuroglobin and Cytoglobin are found in different cell types within the retina and this may relate to the functional oxygen metabolism of those specific cell types.

Maintaining tissue aerobiosis in highly fluctuating hypoxic environments: a hypothetical framework for globin 1 function in *C. elegans*.

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High-affinity myoglobin-like proteins are prevalent in small invertebrates that lack a circulatory system and naturally endure fluctuating or deep hypoxic conditions, such as nematodes, platyhelminths and gastrotrichs. Research on these globins mostly remained confined to *in vitro* biochemical protein analyses with limited *in vivo* research. While providing valuable insights and theoretical predictions, these studies were unable to establish an understanding about the physiological role of these globins in invertebrate oxygen metabolism and responses to hypoxia. **We plan to use the well-characterized and genetically malleable *Caenorhabditis elegans* model to investigate invertebrate globin function with unprecedented functional depth.**

***C. elegans* is an 1 mm-long nematode that thrives in moist, microbe-rich decomposing plant material – an environment characterized by hypoxia – and expresses a high-affinity myoglobin-like protein: GLB-1.** Its capacity to **reversibly bind oxygen** has been well characterized earlier and is substantiated by the position of key amino acid residues essential for this function.

Recent single-cell transcriptomics data revealed that, under normoxic conditions, *glb-1* is lowly expressed in muscles and hypodermis, moderately in certain neurons, and highly in glial tissues. Taking into consideration protein abundances and tissue volumes, we predict that GLB-1 concentrations reach millimolars in glial cells. GLB-1's high oxygen affinity (P_{50}~0.047 torr), dissociation kinetics, and *C. elegans'* oxygen demands, suggest that glial GLB-1 primarily facilitates oxygen diffusion into adjacent neurons rather than serving as an oxygen storage. This raises two fundamental questions: what is the function of an oxygen-supplying protein constitutively expressed during normoxia and why would this function be restricted to glia or neurons? **We hypothesize that glial GLB-1 creates a microenvironment for oxygen-sensitive neurons that, despite low and fluctuating ambient oxygen concentrations, ensures constant access to sufficient oxygen for neuronal aerobic respiration.**

The idea that GLB-1 maximizes aerobic respiration is further supported by multiple independent observations showing that hypoxia consistently and strongly upregulates *glb-1* expression systemically (glia, neurons, muscles, and intestine) in a HIF-1-dependent manner (HIF = Hypoxia Inducible Factor). In very small animals, like *C. elegans*, the absence of a circulatory system was thought to preclude any hypoxia-induced mechanism that improves oxygen delivery to cells. Contrary to this assumption, **we hypothesize that the systemic HIF-1-dependent upregulation of *glb-1* improves oxygen diffusion at the cellular level to reinstall systemic oxygen homeostasis during hypoxia.**

To test these hypotheses, we will first map the spatio-temporal dynamics of *glb-1* expression across a range of oxygen concentrations to identify where and when the worm requires GLB-1. Under chronic hypoxia, we will investigate whether HIF-1 upregulated GLB-1 mitigates hypoxic stress, maintains aerobic energy production, and preserves key organismal functions. Finally, we will specifically delineate the neuronal hypoxic zone with neuron-specific hypoxia markers, and after acute and chronic exposures, we will specifically evaluate the role of glial/neuronal GLB-1 in preserving neuron function using behavioural assays. **This future work aims to broaden our understanding of hypoxia tolerance in small invertebrates, potentially uncovering novel molecular mechanisms in which high-affinity myoglobin-like proteins improve cellular oxygen delivery and maintain aerobiosis in animals without a circulatory system.**

Proton-Driven Conformational Switching in Hell's Gate Globin-I: Kinetic Evidence and Mechanistic Modelling

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Hell's Gate globin-I (HGb-I) from *Methylacidiphilum infernorum* exhibits a reversible transition between hexacoordinate and pentacoordinate haem iron states, modulated by pH and lipid binding [1]. Using stopped-flow spectroscopy, we investigated the kinetics of this coordination change following rapid pH jumps from alkaline to acidic conditions. Both ferric and ferrous forms of HGb-I showed clear pH dependence: the rate of transition to the pentacoordinate state increased markedly with increasing proton concentration.

To rationalise this behaviour, we developed and analysed two mechanistic models. In Model 1, protonation precedes and triggers the conformational change from hexacoordinate to pentacoordinate. In Model 2, a pre-existing equilibrium between the two coordination states is shifted by subsequent protonation. Each model yields distinct predictions for the pH dependence of the observed rate. Experimental data strongly support Model 1, in which protonation drives the structural transition.

This study highlights a rare, temperature-independent proton-sensing mechanism in a globin and offers a kinetic framework for understanding ligand accessibility in extreme environments. The clear preference for one mechanistic model demonstrates the value of coupling experimental kinetics with minimal mathematical modelling to resolve conformational switching pathways in haem proteins.

[1] B. J. Reeder, D. A. Svistunenko, M. T. Wilson. Hell's Gate Globin-I from Methylacidiphilum infernorum Displays a Unique Temperature-Independent pH Sensing Mechanism Utililized a Lipid-Induced Conformational Change. *Int J Mol Sci* **2024,** *25*,

Molecular evolutionary reconstruction of primate muscle O_2 storage capacity reveals potentially semi-aquatic ancestors

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The suggestions of past semi-aquatic lifestyles in extinct giant lemurs, ancestors of New World monkeys (NWM's), and ancestors of modern humans -based on partial fossils or comparative soft and hard tissue anatomy- have been amongst the most controversial claims in primate palaeobiology. Our group has earlier shown that the maximum concentration of the O_2-storing muscle protein myoglobin (Mb) in skeletal muscles of several groups of living mammals is a strong indicator of their underwater endurance or diving capacity. We have further shown that the maximal concentration of Mb is linked to the net surface charge of the protein, which can be modelled from its sequence. Here we use a molecular evolutionary approach to reconstruct the evolution of O_2 storage capacity in ancestral primates based on maximum-likelihood ancestral sequence reconstruction and homology modelling of Mb structure and net surface charge. Our results suggest a temporal increase of Mb net surface charge and hence maximum concentration in ancestral NWM's during their initial divergence in South America ~24 - 17.5 million years ago (Mya). After this period, Mb net surface charge started to decline independently in some, but not all, descendant lineages. Intriguingly, the period of elevated Mb net surface charge overlaps with the mega-wetland Pebas system that originated ~23 Mya due to Andean uplift and covered large parts of a greatly expanded 'Pan-Amazonia' whose break-up ~10 Mya is held responsible for increased speciation/extinction dynamics. By contrast, publicly available Mb sequences from ancient DNA fragments of subfossil giant lemurs or Neanderthal and Denisovan genomes do not support increased muscle O_2-storing adaptations in these groups. In conclusion, our molecular evolutionary results raise the possibility of a semi-aquatic phase with increased diving capacity in ancestral NWM's whose palaeobiology otherwise remains largely unknown due to their notoriously poor fossil record.