Distinctive structural properties of THB11, a pentacoordinate Chlamydomonas reinhardtii truncated hemoglobin with N- and C-terminal extensions

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Abstract
Hemoglobins (Hbs) utilize heme $b$ as a cofactor and are found in all kingdoms of life. The current knowledge reveals an enormous variability of Hb primary sequences, resulting in topological, biochemical and physiological individuality. As Hbs appear to modulate their reactivities through specific combinations of structural features, predicting the characteristics of a given Hb is still hardly possible. The unicellular green alga Chlamydomonas reinhardtii contains 12 genes encoding diverse Hbs of the truncated lineage, several of which possess extended N- or C-termini of unknown function. Studies on some of the Chlamydomonas Hbs revealed yet unpredictable structural and biochemical variations, which, along with a different expression of their genes, suggest diverse physiological roles. Chlamydomonas thus represents a promising system to analyze the diversification of Hb structure, biochemistry and physiology. Here, we report the crystal structure, resolved to 1.75 Å, of the heme-binding domain of cyanomet THB11 (Cre16.g662750), one of the pentacoordinate algal Hbs, which offer a free Fe-coordination site in the reduced state. The overall fold of THB11 is conserved, but individual features such as a kink in helix E, a tilted heme plane and a clustering of methionine residues at a putative tunnel exit appear to be unique. Both N- and C-termini promote the formation of oligomer mixtures, and the absence of the C terminus results in reduced nitrite reduction rates. This work widens the structural and biochemical knowledge on the 2/2Hb family and suggests that the N- and C-terminal extensions of the Chlamydomonas 2/2Hbs modulate their reactivity by intermolecular interactions.

Keywords Truncated hemoglobin · Nitrite reduction · Oligomerization · Pentacoordination · Ligand tunnels

Abbreviations
AEC Anion exchange chromatography
EXAFS Extended X-ray absorption fine structure
FHb Flavohemoglobin
GCS Globin-coupled sensor

Hb Hemoglobin
2/2Hb Hemoglobin with a 2-over-2 helical fold
3/3Hb Hemoglobin with a 3-over-3 helical fold
HBD Heme-binding domain
Mb Myoglobin
NaDt Sodium dithionite
RBC-Hb Red blood cell hemoglobin

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catalyze enzymatic reactions, such as the O$_2$-dependent binding of additional ligands such as hydrogen sulfide, and to their primordial functions were those of enzymes or sensors of molecular oxygen (O$_2$) [2, 3]. In all functional globins, an invariant His residue forms a coordinate bond to the central Fe atom at the proximal site of the heme cofactor, whereas substrates bind to the sixth coordination site of the Fe ion, located at the distal site [4]. Hbs known to date belong to either of two structural sub-classes and are phylogenetically divided into three large families. So-termed 3-on-3 Hbs (3/3Hbs) are built from eight alpha helices, labeled A–H according to their sequential order in animal myoglobin (Mb) and red blood cell hemoglobin (RBC-Hb) [5]. These globins feature a ‘sandwich’ of helices A, E, and F over helices B, G and H. The rather recently discovered so-called truncated hemoglobins (THBs) instead feature a 2-on-2-fold, in which helices B and E lie over helices G and H [6].

3/3Hbs form two families whose globin domains cluster into those of the Mb-like flavohemoglobins (FHbs) and the globin-coupled sensors (GCSs) [2]. FHbs and GCSs are chimeric proteins consisting of a globin domain and flavin adenine dinucleotide- and nicotinamide adenine dinucleotide-binding domains in the case of FHbs [7] or regulatory output domains in the case of GCSs [8]. Both families contain members that only consist of the globin domain, the FHb single-domain globins, and the GCS family protoglobins and sensor single-domain globins [2, 9, 10]. Metazoan and plant symbiotic and nonsymbiotic 3/3Hbs belong to the single-domain globins within the FHb family [2, 11].

Hbs featuring the 2-on-2-fold, referred to as 2/2Hbs in the following, constitute the third Hb family, and are further subdivided into three phylogenetic groups termed group I, II and III, or N, O and P [12, 13]. A small additional group IV (or Q) has recently been suggested [14]. Originally, 2/2Hbs were described as single-domain globins [15]. Increasingly available genomic data, however, revealed 2/2Hbs domains fused to domains with predictable functions, such as protein kinase or nitrate reductase, or to extended N- or C-termini of so far unknown function (note that only THB1 to THB4 have assigned names in the Chlamydomonas genome annotation at Phytozome 12; the nomenclature of THB5 to THB12 follows that in Hemschemeier et al. [27]). THB3 lacks the conserved proximal HisF8 residue, THB5 has four consecutive 2/2Hb domains and THB7 features a split globin domain [28, 29, 31]. The proteins that have been analyzed (mostly) in their recombinant forms (THB1–THB4, THB7, THB8, THB10, THB11) show different biochemical and biophysical properties [27, 29–35], suggesting that they fulfill diverse physiological functions.

THB1, THB2 and THB4 are hexacoordinate Hbs and employ LysE10 as the sixth heme-Fe ligand in both the ferric (heme-Fe$^{III}$) and ferrous (heme-Fe$^{II}$) states [29, 30, 32, 33]. LysE10 is conserved in Chlamydomonas THB10 and THB11, whose electronic absorption spectra indicate hexacoordination in the ferric state, but whose reduced (heme-Fe$^{II}$) forms are pentacoordinate according to UV–Vis spectroscopic and, in case of THB11, extended X-ray absorption

### Introduction

Hemoglobins (Hbs) are iron-protoporphyrin-IX (heme b)-binding proteins that are found in all kingdoms of life [1]. They arose early in evolution, and it has been proposed that their primordial functions were those of enzymes or sensors of molecular oxygen (O$_2$) [2, 3]. In all functional globins, an invariant His residue forms a coordinate bond to the central Fe atom at the proximal site of the heme cofactor, whereas substrates bind to the sixth coordination site of the Fe ion, located at the distal site [4]. Hbs known to date belong to either of two structural sub-classes and are phylogenetically divided into three large families. So-termed 3-on-3 Hbs (3/3Hbs) are built from eight alpha helices, labeled A–H according to their sequential order in animal myoglobin (Mb) and red blood cell hemoglobin (RBC-Hb) [5]. These globins feature a ‘sandwich’ of helices A, E, and F over helices B, G and H. The rather recently discovered so-called truncated hemoglobins (THBs) instead feature a 2-on-2-fold, in which helices B and E lie over helices G and H [6].

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| SEC | Size exclusion chromatography |
| THB | Truncated hemoglobin |
fine structure (EXAFS) analyses [31]. The comparison of the structures of THB1 and THB4 and an analysis of primary sequence patterns suggested to determine hexacoordination in previous studies revealed no clear determinants of LysE10 hexacoordination so far [29]. Notably, a recent study of Chlamydomonas THB1 variants, in which LysE10 was exchanged by other residues, indicates that THB1 has an intrinsic structure favoring heme-Fe bis-coordination [36].

THB10 and THB11 differ from the both ferric and ferrous hexacoordinate Chlamydomonas THB1, -2 and -4 by possessing moderately extended N-termini, and long (312 and 225 amino acids, respectively) C-termini, which might play a role in determining the heme-Fe ligand set. However, recombinant forms lacking these C-termini still show patterns typical for ferrous pentacoordinate Hbs and overall very similar UV–Vis spectroscopic and EXAFS features compared to the full-length proteins [31]. Clearly, more structural data are required to understand the biochemical and, eventually, functional diversification of 2/2Hbs.

Here, we report the first crystal structure of a Chlamydomonas pentacoordinate 2/2Hb, the heme-binding domain (HBD) of Chlamydomonas THB11 in its cyanomet form. The tertiary structure recapitulates the typical 2/2Hb fold, but unusual features are observed, such as a kink in helix E, a tilted heme plane and a tunnel system possibly shielded by Met residues. While our aim was to obtain the structure of the full-length protein including its long C-terminus, we were unable to obtain crystals of any protein variant extending the actual globin domain. As this suggested these protein parts to be flexible or disordered, we analyzed variably truncated THB11 forms and found that both N- and C-termini prompted oligomerization, whereas the HBD was monomeric. Notably, C-terminal truncations resulted in reduced nitrite reduction rates.

Experimental procedures

Production of recombinant THB proteins

Generation of constructs

The C. reinhardtii 2/2Hbs encoded by transcripts Cre14. g615400.t1.2 (THB1) and Cre16.g662750.t1.2 (THB11) were produced in recombinant form as reported before by Huwald et al. [31] (see Tab. S1 and S2 in the supplementary material for details). Length variants of THB11 were generated that lacked the polypeptide extensions N- and C-terminal of the globin domain. These are labeled by the suffixes -N (N-terminal truncation), -C (C-terminal truncation) or -NC (both termini truncated). THB11-C has been described before [31]. Expression vectors were purchased from IBA Lifesciences (https://www.iba-lifesciences.com), namely pASK-IBA3plus for sequences encoding THB1, THB11 and THB11-C, and pASK-IBA5plus for THB11-N and THB11-NC (Tab. S1 in the supplementary material). Sequences expressed from pASK-IBA3plus and pASK-IBA5plus result in proteins equipped with a C-terminal or N-terminal Strep-tag II, respectively. Sequences for pASK-IBA5plus were designed in a way that the Strep-tag II could be cleaved off using TEV protease (Tab. S1, Tab. S2 in the supplementary material). The sequence coding for THB1 was codon-adapted for Escherichia coli K12 and provided by Eurofins MWG GmbH (https://www.eurofins.de), equipped with Bsal recognition sites required for cloning. The DNA sequence is provided in Huwald et al. [31]. The THB11 encoding sequences were amplified from cDNA obtained from the C. reinhardtii wild-type CC-124 mt-[137c] (Chlamydomonas Resource Center, University of Minnesota, MN, USA) as described in Huwald et al. [31], utilizing the oligonucleotides listed in Tab. S2 in the supplementary material. All sequences were cut by Bsal and ligated with Bsal-cut expression vectors. Cloning was done using E. coli strain DH5α MCR grown in LB Broth (Lennox) or on LB Agar (Lennox) from Carl Roth GmbH (https://www.carlroth.com) supplied with 100 µg × ml⁻¹ ampicillin at 37 °C. Liquid cultures were shaken at 180 rpm. The expression constructs were sequenced by the DNA sequencing service at the chair for biochemistry, Biochemistry I, receptor biochemistry, at the Ruhr University Bochum, Germany.

Heterologous protein production in E. coli

Recombinant protein production was done in E. coli strain Rosetta™ (DE3) (F⁻ ompT hsdS₆ (rB⁻ mB⁻) gal dcm (DE3) pRARE (CamR) from Merck (https://www.merckmillerapore.com). Cells were transformed through electroporation using 100 ng of the respective expression vector and grown overnight at 37 °C in liquid LB medium containing 100 µg × ml⁻¹ ampicillin and 25 µg × ml⁻¹ chloramphenicol. For heterologous protein production, 5 ml of the precultures were transferred to 500 ml Terrific Broth (TB) medium (24 g × l⁻¹ yeast extract, 12 g × l⁻¹ tryptone, 4 ml × l⁻¹ glycerol, 0.017 M KH₂PO₄, 0.072 M K₂HPO₄) with 100 µg × ml⁻¹ ampicillin and 25 µg × ml⁻¹ chloramphenicol in 2 l Erlenmeyer flasks. In case of all constructs, the E. coli cultures were shaken at 180 rpm and expression was induced by adding 200 ng × ml⁻¹ anhydrotetracycline (AHT) (IBA Lifesciences), while growth temperature, the OD₆₀₀, at which AHT was added and the expression time were optimized for each protein (Tab. S2 in the supplementary material). Afterwards, the cells were harvested by centrifugation [4 °C, 6200×g, 20 min] and washed with 50 ml buffer W (100 mM Tris–HCl pH 8.0, 150 mM NaCl,
2.5 mM ethylenediaminetetraacetic acid (EDTA)]. Sometimes the cell pellets were frozen at –20 °C before protein purification.

**Purification of THBs**

Recombinant 2/2Hbs were purified by Strep-tag affinity chromatography and additionally by anion exchange chromatography (AEC) and size exclusion chromatography (SEC) when indicated. In all cases, elution fractions that contained protein as indicated by the absorbance at \( \lambda = 280 \text{ nm} \) were collected. Calibration of the column was achieved utilizing the Gel Filtration Markers Kit for Protein Molecular Weights 29,000–700,000 Da (Sigma-Aldrich; [https://www.sigma-aldrich.com](https://www.sigma-aldrich.com)).

**SEC**

Preparatory SEC was performed using a HiLoad Superdex 16/600 200 pg column and buffer W as the equilibration and running buffer. Elution fractions that contained protein were concentrated employing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie-Blue R250 staining according to Laemmli [37]. If not directly used for experiments, protein solutions were snap-frozen in liquid nitrogen and stored at –80 °C.

**Strep-tag affinity chromatography**

After heterologous expression of the 2/2Hb encoding sequences, the E. coli cell pellets were resuspended in 20 ml buffer W and lysed by sonication on ice (six cycles, 25 s each, output energy of 50%) using a Branson Sonifier 250 (Branson Ultrasonics Corporation, [https://www.emersonindustrial.com/en-US/branson/]). Insoluble components were removed by ultracentrifugation (4 °C, 180,000×g, 45 min) and subsequent passing of the supernatant through a 0.2 µm pore size sterile filter. The cleared lysate was applied to a gravity column containing 2 ml of Strep-Tag® Superflow® high-capacity resin (IBA Lifesciences), equilibrated with buffer W. The column was washed with 10 ml buffer W, and then the bound proteins were eluted applying 10 ml buffer E (buffer W containing 2.5 mM desthiobiotin). Recombinant THB1 was judged to be pure after the affinity chromatography step. The remaining proteins were further purified through AEC and SEC.

**AEC**

AEC and SEC (see below) were performed utilizing an Äkta HPLC system and columns from GE Healthcare Life Sciences ([https://www.ge lifesciences.com](https://www.ge lifesciences.com)). A HiTrap Q HP 5 ml column was used for AEC. The buffers of the protein solutions were first exchanged to 25 mM Tris–HCl pH 8.0 employing PD MidiTrap G-25 columns (GE Healthcare Life Sciences). The same buffer was used for equilibrating the column as well as for washing. After washing, proteins were eluted by a linear salt gradient established using the equilibration/wash buffer supplemented with 1 M NaCl. The absorbance at \( \lambda = 280 \text{ nm} \) was recorded to collect fractions that contained protein.

**UV–Vis spectroscopic analyses and determination of nitrite reduction rates**

Steady-state UV–Vis spectra were recorded from \( \lambda = 250 \) to \( \lambda = 800 \text{ nm} \) using the UV-2450 spectrophotometer from Shimadzu ([https://www.shimadzu.com/](https://www.shimadzu.com/)) at a resolution of 1 nm,
a gap width of 2 nm and at a fast sampling rate. Recombinant 2/2Hbs at a concentration of 5–10 µM heme in 700 µl buffer W were prepared in UV-cuvettes micro (BRAND; https://www.brand.de) in an anoxic tent with a nitrogen atmosphere containing ca. 1% molecular hydrogen. All buffers and solutions were also kept under anoxic conditions. Before the cuvettes were taken out of the tent they were sealed with white rubber Suba-Seal® 21 (Sigma-Aldrich). Horse heart Mb (Sigma-Aldrich) (10 µM) was included in all experiments as a control for the set-ups. All samples were kept on ice until just before the UV–Vis spectra were recorded. The heme groups of the proteins were reduced by adding sodium dithionite (NaDt) to a final concentration of 200 µM, and oxidized in the presence of 50 µM potassium ferricyanide. For O2-binding assays, excess NaDt was removed by exchanging the buffer using PD MidiTrap G-25 columns and the cuvette was opened to air. NO binding was analyzed by adding diethylamine NONOate diethylammonium salt (DEA-NONOate; Sigma-Aldrich). A 10 mM solution in buffer W was freshly prepared for each experiment under anoxic conditions and added to a final concentration of 200 µM.

Nitrite reduction rates were determined UV–Vis spectroscopically following the decrease of the Soret peak maxima of deoxyglobins (heme-FeII) upon the formation of ferrous iron-nitrosyl globins. To determine the nitrite reduction rates of the globins under pseudo-first order conditions, the assays were conducted in the presence of NaDt. The deoxy forms were prepared in cuvettes in an anoxic tent on ice as described above. Globin concentrations were adjusted to 5 µM heme in 600 µl of anoxic 50 mM HEPES buffer, pH 7.4, 100 mM NaCl, supplemented with 600 µM NaDt. UV–Vis spectra of these samples were recorded to obtain the initial spectrum for each protein in its deoxy state. Then, anoxic potassium nitrite dissolved in the same buffer was added to the samples to reach final concentrations of 0.25, 0.5, 0.75 and 1 mM using a gas-tight syringe (Hamilton; https://www.hamiltoncompany.com/). The cuvettes were gently shaken and then immediately placed into the UV–Vis spectrophotometer at 20 °C. Absorption at the Soret peak maxima of deoxy Hbs was measured every 3 s until the absorption stayed constant, which usually took about 10 min. Final spectra were recorded to ensure that formation of the ferrous nitrosyl complex had occurred. Each experiment was done in technical triplicates per nitrite concentration, and from at least two independently produced batches of recombinant proteins. The pseudo first order rate constants kobs were obtained in OriginPro from exponential decay fits of the absorption changes in time, and averaged per experiment. Linear fits of the kobs mean values obtained using different nitrite concentrations yielded the bimolecular reaction rates.

**Crystallization, structure determination and structure analysis**

THB11-NC was produced and purified as described above. After the removal of the Strep-tag using TEV protease and a final SEC, the heme group was oxidized applying a ten-fold excess of ferricyanide, which was removed afterwards using PD MidiTrap G-25 columns. The cyanomet (heme-FeIII-CN-) form was then generated by adding a two-fold excess of potassium cyanide (KCN). Screening for crystallization conditions was done applying the sitting drop vapor diffusion approach, utilizing Crystal Phoenix dispenser (Art Robbins Instruments, https://www.artrobbins.com). Mixtures of the protein and precipitant solutions (100 nl:100 nl) were incubated in sitting-drop vapor diffusion plates (Corning® 3550 plates; https://www.corning.com) at 4 °C or 18 °C. Initial crystals were obtained at 1:1 mixtures of protein (19 mg × ml−1 in 15 mM Tris–HCl pH 8.0) with reservoir solutions containing 0.1 M HEPES (pH 6.5–7.5) and 20–30% PEG (6.000–8.000) incubated at 4 °C. Final crystals appeared when mixing 1 µl protein solution (19 mg × ml−1 in 20 mM HEPES, pH 7.0) with 1 µl 0.1 M HEPES pH 7.0 and 21% PEG 6.000 on CrystalClear D Strips (Douglas Instruments, Berkshire, UK) at 4 °C. The crystal was first transferred to a cryoprotectant solution (0.1 M HEPES pH 7.0, 30% (w/v) PEG 8.000) using a micro loop and then snap-frozen in liquid nitrogen. Single-wavelength Anomalous Dispersion (SAD) datasets at the Fe K-edge were collected at beamline X06DA-PXIII (https://www.psi.ch/sls/pxiii) at the Swiss Light Source (SLS, Villigen PSI, Switzerland), applying five 360° sweeps at different chi angles using the PriGo goniometer [40]. Processing and scaling of diffraction data were done using XDS and XSCALE [41]. SHELX [42] within HKL2MAP [43] was used to identify the position of the Fe ion. PHENIX.PHASER and PHENIX.AUTOBUILD [44] were applied to generate the electron density map and the first atomic model based on the primary sequence of THB11-NC. The model was refined using PHENIX.REFINE [44, 45] and manually build using COOT [46]. Tunnels were calculated employing the CAVER 3.0.1 PyMOL plug-in [47] with default settings (minimum probe radius: 0.9, shell depth: 4, shell radius: 3), selecting the heme-Fe as starting point and excluding the CN– ligand. Heme distortions were analyzed by Normal-coordinate Structural Decomposition [48, 49] (https://milptak.w3.umn.edu/ndsl.html), and structural homologues were identified using the heuristic PDB search on the DALI server [50] (http://ekhidna2.biocenter.helsinki.fi/dali/). Multiple primary sequence alignments were done using Clustal Omega [51] (https://www.ebi.ac.uk/Tools/msa/clustalo/). First, the full-length sequences were aligned in order to determine the HBD boundaries. For the final alignment, only the HBD sequences were used as input.
Results

The aim of this study was to obtain structural information on a ferrous pentacoordinate *Chlamydomonas* 2/2Hb, THB11 (gene ID Cre16.g662750), which is one of the algal Hbs that feature extended N- and, particularly, C-termini of yet unknown function. To investigate the role(s) of these termini, we created recombinant length variants of THB11 lacking either one or both termini, namely THB11-N, THB11-C and THB11-NC, lacking the N-terminal 41 amino acids (-N), the C-terminal 225 residues (-C) or both (-NC). We have compared THB11 and THB11-C before, and the truncation of the C terminus hardly affects steady-state ligand binding and the geometry of the active site as studied by UV–Vis spectroscopic and EXAFS analyses [31]. THB11-N and THB11-NC also bound heme stably, and the UV–Vis spectra of the oxidized (FeIII), reduced (FeII), oxy (FeII–O2) and ferrous nitrosyl (FeII–NO) forms were very similar to those reported before for THB11 and THB11-C [31] (Fig. S1 in the supplementary material). We attempted to crystallize all THB11 length variants, however, despite extended screenings for suitable crystallization conditions, only THB11-NC, which is reduced to the heme-binding domain (HBD), and from which the *Strep*-tag had been removed, formed crystals.

The THB11-NC tertiary structure recapitulates the typical 2/2Hb fold

THB11-NC crystallized in space group *P* 2₁ 2₁ 2₁, with one molecule in the crystallographic asymmetric unit. The structure could be refined to a resolution of 1.75 Å (Table 1) (PDB entry: 6TD7). Eleven N-terminal (GSAGTSTATNA; note that the residues GSA are part of the TEV cleavage site) and five C-terminal (AAEEA) amino acid residues could not be resolved. Six residues (Glu29, Arg54, Ser58, Leu109, Met123 and Leu124; the numbering follows the sequence of the polypeptide whose structure was resolved) were modeled to be present in two conformations. Both the heme cofactor and the CN– ligand could be placed in clear electron density (Fig. S2 in the supplementary material).

The tertiary structure of the THB11 HBD is mostly typical for group I 2/2Hbs [6, 25]. It features two antiparallel helix pairs, BE and GH, surrounding the heme cofactor, a short A- and an absent D-helix, and a long EF-loop (Fig. 1a). The four best-matching structures (Z scores of 17.7, 16.9, 16.8 and 15.6) of globins in the cyanomet form detected by the DALI server [50] were THB1 from *Chlamydomonas* [32, 36] (PDB 6CII), cyanoglobin (GlbN) from *Synechocystis* PCC 6803 [52] (PDB 1S69), Li637 from *Chlamydomonas eugametos/moewusii* [6] (PDB 1DLY) and GlbN from *Mycobacterium tuberculosis* [53] (PDB 1RTE). The higher structural similarity of THB11-NC to THB1 and cyanoglobin

| Table 1 | Data collection, processing and refinement statistics |
| --- | --- |
| Data collection |  |
| Beamline | SLS-X06DA |
| No. of scaled sweeps | 5 |
| Resolution (Å) | 38.54–1.75 (1.81–1.75) |
| Cell parameters |  |
| a, b, c (Å) | 31.04, 42.62, 90.26 |
| α, β, γ (°) | 90, 90, 90 |
| Space group | *P* 2₁ 2₁ 2₁ |
| Wavelength (Å) | 1.739 |
| No. of observations | 594,012 (4,429) |
| Anomalous pairs merged |  |
| No. of unique reflections | 11,933 (711) |
| Completeness (%) | 94.3 (58.4) |
| Multiplicity | 49.8 (6.2) |
| Average I/σ I | 38.4 (2.6) |
| CC (1/2) | 1.0 (0.82) |
| Rmerge | 9.5 (56.6) |
| Rfree | 9.6 (61.7) |
| Anomalous pairs unmerged |  |
| No. of unique reflections | 21,945 (711) |
| Completeness (%) | 94.3 (58.4) |
| Multiplicity | 27.1 (3.3) |
| Average I/σ I | 28.4 (1.9) |
| CC (1/2) | 1.0 (0.73) |
| Rmerge | 8.8 (52.6) |
| Rfree | 8.9 (61.9) |
| Wilson B-factor (Å²) | 17.99 |
| Phasing |  |
| Resolution for phasing (Å) | 1.75 |
| Iron sites | 1 |
| Figure of merit | 0.35 |
| Refinement |  |
| Rwork/Rfree | 0.173 / 0.210 |
| Root-mean-square deviation bond length (Å) | 0.005 |
| Root mean square deviation angles (°) | 0.893 |
| Ramachandran favored (%) | 99 |
| Ramachandran outlier (%) | 0 |
| Average B-factor (Å²) | 18.9 |
| Protein (Å²) | 17.6 |
| Ligand (Å²) | 19.3 |
| Solvent (Å²) | 26.9 |
| No. of atoms (without riding hydrogens) | 1,260 |
| Protein | 1,062 |
| Ligands | 45 |
| Water | 153 |
| PDB code | 6TD7 |

Values in parenthesis represent the data for the highest resolution shell

*SLS* Swiss Light Source
is reflected in the F-helix, which is comprised of only one helical turn in the 2/2Hbs from C. eugametos and M. tuberculosis, but of about three turns in the former three proteins (Fig. S3 in the supplementary material). THB11-NC differs from other class I 2/2Hb structures by featuring a noticeable kink in the E-helix (Figs. 1a, S3).

The active site displays a moderate ligand-stabilizing H-bond network

Only one heme orientation isomer could be fitted into the electron density map of THB11-NC, and the porphyrin ring is rotated by 180° along the α-γ axis compared to those of THB1, LI637 and Synechocystis or M. tuberculosis GlbN. Compared to those of additional 2/2Hbs, the heme plane is notably tilted when inspecting the THB11 HBD structure from the side of the A-helix (Fig. 1b). Normal-coordinate structural decomposition analysis [48, 49] indicates that the heme group of THB11-NC features an overall out of plane displacement of 0.49 Å, and the heme distortions are dominated by doming and ruffling. The distances between the Fe ion and the porphyrin N-atoms are 2.1 Å (pyrrole rings A and D) and 2.0 Å (pyrrole rings B and C), and 2.2 Å to both the HisF8 Nε atom and the CN– ligand (Fig. 2a, c). In Hbs, viewed from the top, the imidazole plane of HisF8 can lie parallel to the line that connects opposite pyrrole ring N atoms of the porphyrin ring system, or form an angle of about 45°. These positions are referred to as eclipsed and staggered, respectively. The imidazole ring of THB11-NC HisF8(81) (note that residues are indicated in the Perutz nomenclature, followed by their numbers in the structure in brackets) is in a moderately staggered position, and its plane is tilted towards the heme group (Fig. 2a, c). The Nδ-atom of HisF8(81) is in moderate H-bond distance (2.6 Å) to the backbone carbonyl of ValF4(77), while the heme propionates form polar contacts (2.3 Å to 3.3 Å) to ArgF10(83), Lys75 in the EF-loop and five water molecules (Fig. 2a, b).

In group I 2/2Hbs, polar residues at the topological positions B10, E7 and/or E11 have been shown to contribute to ligand stabilizing H-bond networks [25], and references therein [see an alignment of several Chlamydomonas 2/2Hb HBDs to additional 2/2Hbs in Fig. S4 in the supplementary material]. In the distal site of THB11-NC, the CN– ligand lies in moderate H-bond distance to the hydroxyl group of TyrB10(32) (2.8 Å), whereas position E7 is occupied by Leu53, and the distance of the ligand to the amide group of GlnE11(57) is 3.9 Å (N atom) or 4.9 Å (O atom). The TyrB10(32) hydroxyl group itself is in moderate to weak H-bond distance to GlnE11(57), namely 3.0 Å (O atom) and 3.5 Å (N atom), respectively (Fig. 2c). LysE10(56) points away from the heme cofactor, being located at the E helix side that is averted from the distal niche.
The THB11-NC crystal structure indicates an uncommon tunnel system

Group I 2/2Hbs have protein cavities or tunnels involved in ligand migration and accumulation. In *M. tuberculosis* GlbN, a longer tunnel (LT) is positioned roughly perpendicular to the heme plane and exits the protein between the hinges of the A- and B- and the G- and H-helices. A shorter tunnel (ST) runs approximately parallel to the heme plane and exits the protein between helices G and H [21, 54] (Fig. 3a). Similar L-shaped tunnel systems have been described for additional class I 2/2Hbs, such as that of *Tetrahymena pyriformis* and *C. eugametos* [21, 55]. In *Synechocystis* and *Synechococcus* GlbNs, ligand exchange might also occur at the solvent exposed heme edges [52, 56, 57]. In the *Chlamydomonas* THB11-NC crystal structure, the LT, but not the ST could be detected by Caver, and a tunnel exiting at the solvent exposed heme edge was computed (Fig. 3a).

In *M. tuberculosis* GlbN, the LT opens to the solvent by an aperture formed by IleA15, IleB2, ValB5, and LeuG16, and the ST entry is formed by PheG5, AlaG9, LeuH8, IleH11 and AlaH12 [21, 54]. In THB11-NC, these positions are at similar topological sites. A15, B2 and B5 are occupied by Val residues 18, 24 and 27, whereas Met106 is at the G16 site (Fig. 3b). PheG5 is highly conserved in class I 2/2Hbs [13, 14] (also see Fig. S4). In THB11-NC, a Phe is also found at the respective site (residue 95), however, the G9, H8, H11 and H12 positions are occupied by Met99, Met120, Met123 and Leu124, respectively (Fig. 3b). Met123 as well as Leu124 were modeled to be present in two conformations (63% and 37% occupancy in both cases). The intersection of the LT and ST is formed by residues E15 and G12, which are both Leu residues in *T. pyriformis* TrHbN (Leu54 and Leu90) [55], whereas Phe62 forms the so-termed E15 gate in *M. tuberculosis* GlbN [54, 58, 59]. In THB11-NC, E15 is represented by Val61. Here, however, the position opposite to the E15 residue is occupied by a bulky Phe residue (PheG12(102)) (Fig. 3b).

The THB11-NC crystal structure indicates an uncommon tunnel system

Despite many trials we were unable to obtain crystals of a *Chlamydomonas* THB11 protein variant containing either N- or C-terminal extensions. This suggests that these termini might be flexible, or that they result in structural heterogeneity within the protein population. We noted that the purification of all THB11 length variants except THB11-NC through anion exchange chromatography (AEC) repeatedly resulted in two well defined elution peaks (Fig. 4a), whose dominant proteins represented the respective recombinant THB11 variant according to their size in denaturing gel electrophoresis (Fig. 4b). The protein solutions from both AEC peak fractions were subsequently tested for possible oligomeric states by native gel electrophoresis and size exclusion chromatography (SEC). All THB11 length variants except THB11-NC were present in more than one band after native gel electrophoresis, whereas the number of bands differed between the variants as well as the two AEC fractions, and was highest in case of full-length THB11 (Fig. 4b). Analytical SEC showed that all protein variants except THB11-NC...
eluted as two major protein peaks (Fig. 4c). According to the calibration of the SEC column, these represented approximately tri- and heptamers in case of THB11 and THB11-N, and mono- and trimers in case of THB11-C, whereas the elution volume of the single THB11-NC peak corresponded to a monomer. The protein solutions obtained from the two AEC elution fractions were represented by different ratios of the two oligomeric states, which was particularly pronounced in case of THB11-C (Fig. 4c). UV–Vis spectra of the THB11 length variants present in the different AEC peaks were almost identical in all cases and represented the oxy forms (FeII–O2) (Fig. 4d).

The THB11 C terminus influences nitrite reduction activities

As noted before for THB11 and THB11-C [31] and shown here also for THB11-N and THB11-NC (Fig. S1 in the supplementary material, Fig. 4d), steady-state UV–Vis spectra of the length variants in different states and in the presence or absence of ligands are very similar. To test whether the termini of THB11 exert an influence on the kinetics of a reaction, we determined the nitrite reduction rates of the protein variants. Nitrite reduction to NO by the deoxy (heme-FeII) forms of globins [60] has been suggested to be a potential in vivo function especially of globins from photosynthetic organisms [61, 62] (also see discussion), and the kinetics of this reaction can be traced robustly by UV–Vis spectroscopy. The reaction can be described as Hb-FeII + NO2− + H+ → Hb-FeIII + NO + OH−, followed by a rapid binding of NO to additional deoxylobins (Hb-FeII + NO → Hb-FeII–NO). Thereby, in the absence of reducing agents, two deoxyglobins react with nitrite to one iron-nitrosyl globin (Hb-FeII–NO) and one metglobin (Hb-FeIII), whereas in the presence of a reductant such as NaDt, Hb-FeIII is rapidly re-reduced and deoxyglobin is completely converted to Hb-FeII–NO [63]. Here, nitrite reduction rates were thus analyzed by incubating the deoxy (FeII) forms with varying concentrations of nitrite in the presence of excess reductant (600 µM NaDt), resulting in pseudo-first order reaction kinetics (Fig. S5 in the supplementary material shows exemplary kinetic traces and the averaged pseudo first order rate constants  kobs). Horse heart Mb as well as Chlamydomonas recombinant THB1 served as controls, and their nitrite reduction rates of 4.7 ± 0.6 M−1 s−1 (Mb) and 45.9 ± 2.7 (THB1) (Fig. 5) correlated with reported rates (Mb: e.g. 2.9 M−1 s−1 [64] or 5.5 M−1 s−1 [65]), determined at pH 7.4 and pH 7.41, respectively; THB1: 98 M−1 s−1, determined at pH 6.6 [30]). Full-length THB11 had a nitrite reduction rate of 25.9 ± 2.6 M−1 s−1, which was similar to the rate observed for THB11-N (24.9 ± 6.1 M−1 s−1), but higher than those of both C-terminally truncated variants, which were 13.4 ± 2.1 M−1 s−1 (THB11-C) and 17.1 ± 2.9 M−1 s−1 (THB11-NC) (Fig. 5).
Fig. 4 THB11 length variants form different oligomeric states. 

(a) Anion exchange chromatography (AEC) profiles of the indicated protein variants, which had been purified from E. coli cell lysates by Strep-tag affinity chromatography before. The brackets above the peaks indicate the elution fractions that were combined for subsequent analyses [black, blue: peak (P) 1, 2]. The molecular mass of each protein calculated from the primary sequence are indicated to the left.

(b) Denaturing (SDS) and native gel electrophoreses of the protein solutions collected from the AEC peaks P1 and P2. The numbered bands observed in the native gels were cut out and subsequently subjected to denaturing gel electrophoresis.

(c) Size exclusion chromatography (SEC) profiles of the proteins present in the two AEC peaks. kDa labels within the SEC graphs indicate the molecular masses calculated from the elution volumes according to the column’s calibration curve, colors indicate the original AEC P1 (black) or P2 (blue).

(d) UV–Vis spectra of the protein solutions collected from the two AEC peaks, P1 and P2. The spectra were normalized with regard to the Soret maxima (set to 1)
The characterization of the 12 class I 2/2Hbs of Chlamydomonas is at an early stage. The available physiological, transcript level and biochemical data speak for diverse physiological functions and fine-tuned protein characteristics [29, 31, 33, 35, 66–68]. THB8 to THB12 form a group of algal 2/2Hbs that feature moderately elongated N- and considerably elongated C-termini, extending the HBD by about 50 N-terminal and 225 (THB11) to 536 (THB9) C-terminal amino acids. THB10 and THB11, two members of this group that have been studied in their recombinant holo forms, are pentacoordinate in the reduced state (heme-Fe$^{III}$), whereas the single-domain algal globins THB1,-2 and -4 are hexacoordinate [29, 30, 33]. Although the role of hexa- versus pentacoordination, found in both 2/2Hbs and 3/3Hbs, is still unclear, the different structure–function relationships observed in hexa- and pentacoordinate globins indicate specialized physiological functions [23]. Here, we solved the crystal structure of the THB11 HBD, in order to widen the structural knowledge base for understanding the biochemical, and, ultimately, physiological functions of the versatile 2/2Hbs from Chlamydomonas. The tertiary structure of the THB11 HBD recapitulates the overall 2/2Hb topology well (Figs. 1, S3), but it reveals several features that are likely to result in specific reactivities. These are the noticeable kink in helix E, the tilted heme plane, a distal site of intermediate polarity, and an unusual accumulation of Met residues at topological sites known from other class I 2/2Hbs to form tunnel openings.

The tilt of the heme plane appears to be a unique feature of THB11-NC when compared to additional class I 2/2Hbs (Figs. 1b, S3), and might be related to the kink in helix E. Although this particular conformation might be an artificial consequence from the rigid crystal packing, and/or a result of the deletion of the N- and C-termini, the observed heme tilting might contribute to the marked asymmetry of the Fe center we observed by EXAFS analyses of the deoxy (Fe$^{III}$) and oxy forms (Fe$^{III}$-O$_2$) of full-length THB11 as well as THB11-C [31]. Overall, the distances between the heme-Fe and surrounding first, second and third sphere atoms measured in the cyanomet THB11-NC crystal structure are comparable to the EXAFS simulation parameters obtained with oxy full-length THB11 and THB11-C (Fig. S6 in the supplementary material), suggesting that neither crystallization nor the removal of the termini resulted in large deviations from the actual protein structure in solution. The kink in the E-helix might be related to the absence of the Gly–Gly motif present in many class I 2/2Hb primary sequences directly following the residues forming helix E [13, 14] (Fig. S4 in the supplementary material). Together with Gly motifs after the A- and preceding the F-helix, these are thought to provide the flexibility required to stabilize the truncated 2/2Hb fold [25]. In the THB11 primary sequence, the E-helix is followed instead by a Gly–Pro–Pro motif, which is likely to result in a strongly decreased flexibility of the EF-turn.

The heme tilt observed in THB11-NC goes along with a leaning of the HisF8 imidazole plane towards the porphyrin, a conformation that is related to a lower O$_2$ affinity in hemoglobin α [4], and references therein]. On the other hand, the THB11 HisF8 plane is in a moderately staggered position, which has been associated with a higher O$_2$ affinity, especially when the Nδ of HisF8 forms a H-bond to a carbonyl group [69], which is the case in THB11-NC (Fig. 2a, b). Additionally, distortions of protein-bound heme groups from an optimally planar and symmetric system are common [70] and can play major roles in determining the reactivity of the heme–iron, such as ligand affinity and redox potentials [71–73]. With an out of plane displacement of about 0.5 Å, the heme group of THB11-NC features a small heme distortion compared to other class I 2/2Hbs [74]. Its dominant modes, doming and ruffling, are often observed among this class of truncated hemoglobins, although ruffling seems to be slightly more common [74]. While we did not determine ligand/substrate affinities for THB11 and its length variants, we noted that all proteins were consistently purified in their oxy forms. These states were stable throughout the purification process (e.g.
see Fig. 4d), suggesting that the O₂ ligand was well stabilized by all protein variants. This notion is supported by a preliminary determination of autoxidation rates. These vary between 0.004 h⁻¹ (THB11-N) and 0.01 h⁻¹ (full-length THB11) and were thus comparable to the autoxidation rate of Mb [75]. H-bond networks that stabilize heme-bound ligands and result in low ligand dissociation rates are observed frequently in the distal site of group I 2/2Hbs. TyrB10 is the most conserved [13, 14] and forms a direct H-bond to the heme-bound ligand [6, 53, 76, 77]. In several cases, the ligand also forms H-bonds to GlnE7, such as in C. eugametos, Paramaecium caudatum and T. pyriformis 2/2Hbs [6, 55], whereas TyrB10 is additionally stabilized by H-bonds to a polar E7 and/or E11 residue [6, 53, 55]. A polar E7 residue is not essential for a strong ligand stabilization, as shown for M. tuberculosis GlbN, which features LeuE7, but a high O₂ affinity and a low autoxidation rate [76]. In the THB11 HBD crystal structure, the CN⁻ ligand is in a strong H-bond distance only to TyrB10(32), which, in turn, is in moderate H-bond distance to GlnE11(57), similar to the situation in M. tuberculosis GlbN [54, 76, 78]. In M. tuberculosis cyanomet GlbN, however, the CN⁻ ligand is additionally in weak H-bond distance (3.4 Å) to GlnE11 [53], whereas this distance is at least 4.0 Å in THB11-NC, which might result in a less pronounced stabilization of a given ligand, consistent with autoxidation rates five- to ten times higher than in M. tuberculosis GlbN [76].

The reactivity of a given globin is also strongly dependent on access and escape routes through which ligands and substrates/products can enter or escape. In many 2/2Hbs, tunnels and cavities have been observed such as the L-shaped system of a long (LT) and a short tunnel (ST) in the class I 2/2Hbs from M. tuberculosis, T. pyriformis and C. eugametos [21, 54, 55] (also see Fig. 3a). The tunnels and cavities are dynamic and dependent on the presence or absence of ligands. In the crystal structures of the class I hexacoordinate 2/2Hbs from Synechocystis and Synechococcus, for example, access tunnels are only observed when a ligand is bound to the heme-Fe [52, 74]. In case of M. tuberculosis GlbN, which has a high nitric oxide (NO) dioxygenase activity, it is proposed that O₂ enters the protein through the ST, and that binding of the O₂ molecule to deoxy-GlbN triggers the opening of the LT through a gating mechanism operated by PheE15 [58, 59, 79, 80]. Thus, the missing ST in THB11-NC has to be interpreted with caution, and ligands might simply enter or leave through the exposed heme edge in any case. However, it is noteworthy that the LT can be computed within the THB11 HBD structure (Fig. 3a), and that the residues that form the LT aperture in M. tuberculosis and T. pyriformis class I 2/2Hbs are similar in THB11 [21, 54, 55] (Figs. S4, S7 in the supplementary material). In contrast, the clustering of Met residues at the possible ST aperture as observed in THB11 appears to be unusual. These positions (PheG5, AlaG9, LeuH8, IleH11 and AlaH12 in M. tuberculosis GlbN) are mostly aliphatic in other class I 2/2Hbs, whereas several of the algal Hbs, including THB1, possess Met residues at one or the other position [13] (Figs. S4, S7). The H11 site has been implicated to be of importance for ligand access by computational approaches [14]. IleH11 is indeed shifted in the isobutyl cyanide derivative of M. tuberculosis GlbN, and both IleH11 and AlaG9 are shifted in the xenon adduct, causing the ST entrance to widen [21]. In the THB11-NC structure, MetH11(123) as well as the adjacent LeuH12(124) were modeled in two conformations, and, together with MetG9(99), MetH11(123) restricts the site at which the ST exits in M. tuberculosis GlbN (Fig. 3).

In silico mutagenesis of both residues (M99A and M123I) resulted in a model in which an ST very similar to that of M. tuberculosis GlbN was computed by Caver (Fig. S8 in the supplementary material).

It is tempting to speculate that the Met clustering could play a particular role for the regulation of ligand access in THB11, especially in the context of an algal host that photosynthetically produces reactive oxygen species on a regular basis. Met residues are prone to oxidation, a process that is reversible by methionine sulfoxide reductases and employed for antioxidative defense as well as for protein activity regulation [81]. We do not know where the THB11 C terminus would be located and if it would shield the ST access site, respectively. Also, most of the Met residues found at the putative ST aperture in THB11-NC are buried within the protein. However, depending on the surrounding flexibility, even buried Met side chains can be oxidized [82], and both MetG9(99) and MetH8(120) are close to the surface.

Structurally, Met residues are often involved in hydrophobic interactions. From the THB11 HBD crystal structure, we cannot infer the positions of the N- and C-termini relative to the HBD. However, it is noteworthy that the C terminus consists of hydrophobic amino acids to almost 70%. It is very Ala-rich (about 36%) and contains two Phe, one Trp and one Tyr. The latter three residues are often involved in stabilizing Met-aromatic motifs [83]. It is possible that these amino acids confer interactions of the termini (the N terminus also contains one Phe and one Trp each) to the HBD, in whose structure, as noted above, the putative ST aperture residues MetG9(99) and MetH8(120) are close to, and Met51 and Met59 are directly at the surface. Met-aromatic interactions might also confer the intermolecular interactions we infer from analyses of the oligomeric states of the THB11 length variants containing at least one of the termini (Fig. 4), but these might also or additionally be promoted by other hydrophobic interactions or by disulfide bridges, as both termini contain one Cys each.

A physiological relevance of the THB11 differential oligomerization remains to be proven, especially as the in vivo role of THB11 has not been investigated yet. THB11 is not
predicted by the algae-specific localization prediction tool PredAlgo [84] to be localized in an organelle, whereas peptides of THB11 have been detected in the mitochondria-enriched fraction [85]. THB11 transcript levels are higher in *Chlamydomonas* cells in the dark [31, 86] and increase steadily in the subjective night in synchronized algal cultures [66, 87], when also THB11 peptides can be detected [87]. These data might suggest a differential function of THB11 in the night versus the day, and the different intracellular metabolic states present under these conditions might well influence the oligomerization status of the protein. This, in turn, might influence reaction kinetics, as the absence of the C-terminus results in a reduction of the nitrite reduction rate by about 50% (Fig. 5). Although our current data do not allow us to distinguish between a role of the C-terminus by itself or by its influence on the oligomerization status, extensions of the globin domain have been shown before to influence the reactivities of globins, such as the pre-A motif of *M. tuberculosis* GlbN, which is crucial for the high NO dioxygenase activity of the protein. It has been proposed that its absence influences protein dynamics in a way that the PheE15 gate constitutively blocks the long tunnel [88], whereas more recent results suggest that the pre-A motif prevents the formation of stable GlbN dimers, in which the short tunnel is blocked, and the long tunnel restricted [89]. In *Methanosarcina acetivorans* protoglobin, a 3/3Hb, dimerization has instead been calculated to result in an opening of the tunnels [90]. The single class II 2/2Hb from *A. thaliana* has comparably short N- (25 residues) and C-terminal (32 residues) extensions, however, these appear to modulate its oligomeric state (both termini), the coordination of its heme-Fe (N-terminus) as well as azide binding to its ferric state (C-terminus) [91].

To date, although many different Hb types have been shown to catalyze nitrite reduction to NO, generalizable properties that are rate-determining are not known. Factors that have been discussed are hexa- versus pentacoordination, H-bond donors in the distal site, distal site volume, the redox potential, and hydrophobic cavities and tunnels [60, 64, 92, 93]. Noteworthy, the importance of a given structural or biochemical element appears to vary in different globin types. For example, pentacoordinate variants of the naturally hexacoordinate 3/3Hb neuroglobin exhibit strongly increased activities [64, 93], whereas a similar mutation in *Arabidopsis* class I nonsymbiotic Hb has hardly any effect [92]. In general, as has also been discussed by Sturms et al. [61], hexa- or pentacoordination is not suitable to predict nitrite conversion velocity, since the groups of penta- and hexacoordinate Hbs both contain high- and low-efficiency nitrite reductases.

The nitrite reduction rates of *Chlamydomonas* THB11 and its variants were higher than those of most animal Hbs studied at similar pH values, for which rates from 0.062 M$^{-1}$ s$^{-1}$ (human neuroglobin with reduced cysteine residues; Tiso et al. [64]) to 6 M$^{-1}$ s$^{-1}$ (human RBC-Hb in the R-state; Huang et al. [94]) have been reported [see a compilation of rates in [60]]. THB11 is thus more comparable to algal, plant and bacterial Hbs with reported nitrite reduction rates of ca. 46 M$^{-1}$ s$^{-1}$ (this study) or 98 M$^{-1}$ s$^{-1}$ [30] for *Chlamydomonas* THB1, 54 M$^{-1}$ s$^{-1}$ for THB2 [30], 19 M$^{-1}$ s$^{-1}$ and 83 M$^{-1}$ s$^{-1}$ for *Arabidopsis* and rice class I nonsymbiotic Hbs (3/3Hbs), respectively, or 68 M$^{-1}$ s$^{-1}$ for *Synechocystis* GlbN [61, 62]. As explained above, it is hardly possible to ascribe the comparably high nitrite reduction rate to any given feature of THB11, although the putative H-bond stabilizing network in the distal site and the observed (LT, heme edge) or suggested (ST) access routes might contribute to efficient ligand stabilization and access or escape. Likewise, we do not know whether nitrite reduction by globins plays a physiological role in *Chlamydomonas*, and if so, which. It has been suggested that the comparably high nitrite reduction activities observed for several Hbs from photosynthetic organisms might be employed for nitrite detoxification and/or for NO generation [61, 62]. Within the cellular environment, it is relevant that Hb-catalyzed nitrite reduction requires the ferric heme to be re-reduced to its ferrous form. It may be in this context that the ferric hexacoordination of THB11 indicated by UV–Vis spectroscopy plays a role, because the presence of a sixth ligand can influence both the redox potential and reduction kinetics of a globin [95]. In any case, an adequate reductase or reducing equivalent must be present. *Chlamydomonas* THB1, -2 and -4 can be reduced by both the diaphorase domain of nitrate reductase and by a ferredoxin – ferredoxin:nicotinamide adenine dinucleotide phosphate reductase system [29, 35], but this has not been tested for the additional algal Hbs. Still, nitrite reduction to NO by (one of) the algal 2/2Hbs might be one route of NO production in *Chlamydomonas*. Whereas nitrate reductase has been shown to be involved in NO generation in the alga [96, 97], many laboratory strains are deficient for this enzyme. These strains still appear to employ NO-based signaling pathways [27, 98–100], and nitrite as a source for NO production has been suggested [99, 100]. Because NO usually binds to deoxyglobins with a high affinity, a role of globins in free NO generation is still controversial. However, free NO accumulates upon nitrite reduction by *Arabidopsis* Hbs [62]. Also, it has been shown that deoxy-Mb, in the presence of nitrite, can inhibit mitochondrial respiration [101], and NO production from nitrite by cytoglobin was correlated with a higher activity of NO-sensitive guanylate cyclase [102].

In summary, we have solved the structure of the HBD of one of the ferrous pentacoordinate *Chlamydomonas* 2/2Hbs. THB11, demonstrating several unique features
that are likely to result in a specific function of the protein. The cellular role of the extended termini remains to be investigated, but our studies on THB11 length variants show that they prompt oligomerization in vitro, suggesting that they confer protein–protein interactions also in the cell. The effect of the absence of the C terminus on nitrite reduction rates indicates that it can regulate THB11 activity, which is the first hint for a function of the algae-specific extensions of a conserved 2/2Hb core.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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