Thr-E11 Regulates O\textsubscript{2} Affinity in Cerebratulus lacteus Mini-hemoglobin*

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The mini-hemoglobin from Cerebratulus lacteus (CerHb) belongs to a class of globins containing the polar Tyr-B10/Gln-E7 amino acid pair that normally causes low rates of O\textsubscript{2} dissociation and ultra-high O\textsubscript{2} affinity, which suggest O\textsubscript{2} sensing or NO scavenging functions. CerHb, however, has high rates of O\textsubscript{2} dissociation (k\textsubscript{O\textsubscript{2}} = 200–600 s\textsuperscript{-1}) and moderate O\textsubscript{2} affinity (K\textsubscript{O\textsubscript{2}} ~1 \textmu{M}\textsuperscript{-1}) as a result of a third polar amino acid in its active site, Thr-E11. When Thr-E11 is replaced by Val, k\textsubscript{O\textsubscript{2}} decreases 1000-fold and K\textsubscript{O\textsubscript{2}} increases 130-fold at pH 7.0, 20 °C. The mutation also shifts the stretching frequencies of both heme-bound and photodissociated CO, indicating marked changes of the electrostatic field at the active site. The crystal structure of Thr-E11 → Val CerHbO\textsubscript{2} at 1.70 Å resolution is almost identical to that of the wild-type protein (root mean square deviation of 0.12 Å). The dramatic functional and spectral effects of the Thr-E11 → Val mutation are due exclusively to changes in the hydrogen bonding network in the active site. Replacing Thr-E11 with Val “freezes” the Tyr-B10 hydroxyl group to rotate toward and donate a strong hydrogen bond to the heme-bound ligand, causing a selective increase in O\textsubscript{2} affinity, a decrease of the rate coefficient for O\textsubscript{2} dissociation, a 40 cm\textsuperscript{-1} decrease in ν\textsubscript{CO} of heme-bound CO, and an increase in ligand migration toward more remote intermediate sites.

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Globins are found in all kingdoms of living organisms. Their functions have been the subject of active debate. In addition to O\textsubscript{2} transport and storage (1–3), several novel functions have been proposed recently, including control of NO levels in microorganisms and nerve tissue, O\textsubscript{2} sensing, and dehaloperoxidase activity (4–8). Nerve tissue Hbs are found in both vertebrates and invertebrates. Neuroglobin is a recently discovered member of the globin family, whose in \textit{vivo} function is still unknown (9–14). It is expressed in specific regions of vertebrate brains, displays low sequence identity to conventional Hbs or Mb,\textsuperscript{1} and is characterized by a bis-His-Fe hexacoordinate heme structure (11, 12, 15, 16). In contrast, the nerve tissue Hbs found in mollusc, annelid, arthropod, nemertean, and nematode species (17, 18) appear to store and/or transport O\textsubscript{2} to support brain and axon function during temporary hypoxia (18–21).

The nerve tissue and body wall Hbs of the nemertean worm \textit{Cerebratulus lacteus} (CerHb)\textsuperscript{2} are the smallest naturally occurring Hbs, composed of only 109 amino acids. Analysis of the three-dimensional structure of nerve tissue CerHb has shown that the typical 3-over-3 globin fold is edited markedly (22). The N-terminal A-helix is deleted; the GH region is extended; and the C-terminal H-helix is shortened. Both sequence and fold comparisons suggest that CerHb is equally distant from all known globin tertiary structures, supporting its identification with a new superfamily, the mini-Hbs (22). CerHb contains a large elongated tunnel in its interior. Ligands may enter and exit CerHb through this apolar tunnel between the E- and H-helices, whose solvent access is made easier by the absence of the A-helix, the presence of small residues lining the cavity, and the shorter spans of some of the individual helices (22). Similar apolar cavities/tunnels are observed in a variety of heme-containing enzymes that react with gases or apolar ligands (23).

The ligand-binding site of both body wall and nerve CerHb is highly unusual since it contains three polar residues, Tyr-B10, Gln-E7, and Thr-E11. Normally, Hbs that contain the Tyr-B10 and Gln-E7 side chains show enhanced O\textsubscript{2} affinity and mark-

\textsuperscript{1} The abbreviations used are: Hbs, myoglobin; CerHb, \textit{C. lacteus} mini-hemoglobin; FTIR, Fourier-transformed infrared spectroscopy; TDS, temperature derivative spectroscopy; r.m.s.d., root mean square deviation; wt, wild-type.

\textsuperscript{2} CerHb amino acids have been identified with their topological site numbers as defined in the conventional globin fold.
edly reduced rates of dissociation due to strong hydrogen bonds between these polar amino acids side chains and the heme-bound ligand (24, 25). The Tyr-B10 hydroxy group and Gln-E7 N-ε atom in wild-type nerve CerHb are very close (3.0 Å) to the heme-bound O₂ atoms, indicating significant electrostatic interactions (22). In addition, the imidazole ring of the proximal histidine adopts a staggered orientation with respect to the pyrrole N atoms in CerHbO₂, a conformation that allows in-plane movement of the iron atom with minimal steric hindrance. Leghemoglobins have similar staggered proximal geometric orientation with oxygen usage optimized for all ligands (26, 27). In contrast, the affinity of CerHb for O₂ is moderate (P₅₀ = 0.6 mm) and the rate coefficient for O₂ dissociation is understandably large, 200–600 s⁻¹ (Table 1).

To resolve this apparent discrepancy between structure and observed functional properties, we have surveyed the effects of point mutations of the three polar amino acids in the active site of nerve CerHb (Tyr-B10, Gln-E7, and Thr-E11) on O₂ binding. The largest effects were observed when Thr-E11 was changed to a small, apolar amino acid like Val. The consequences of this replacement on structure and function were examined in detail by determining the crystal structure of the Thr-E11 → Val CerHbO₂ mutant, measuring overall rates of O₂ and CO binding to wild-type and mutant CerHbs, and examining the IR spectral properties of the corresponding heme-bound and photodissociated CerHbCO complexes.

**Materials and Methods**

*Measurement of Overall Rates of Ligand Association and Dissociation at Ambient Temperature—Wild-type and mutant recombinant CerHbs were expressed and purified as described previously using a synthetic genetic gene with codon usage optimized for expression in *Escherichia coli* (22). The native body wall CerHb was isolated from whole animals and purified as described (18). Most of the recombinant nerve CerHb and also the body wall CerHb sample were isolated in the reduced state and used directly. Samples containing significant amounts of ferric CerHb were reduced by dithionite and run a G-25 column to remove excess reducing agent. All experiments were carried out in 0.1 M phosphate buffer, pH 7.0, 2.0 mm EDTA, 20°C. The sample solutions were drawn into a gas-tight syringe and inserted into a sealed cuvette (1 mm path length), previously equilibrated with the appropriate O₂ partial pressure. O₂ association time courses were measured after complete laser photolysis of CerHbO₂ samples, using a 300-nm excitation pulse from a Phase-R model 2100B dye laser (28). Simple exponential rebinding time courses were observed for most of the samples, and k₁O₂ was determined from the slope of kobs versus [O₂]. The association rate coefficient for NO binding to wild-type CerHb was measured in the same way. Cuvettes were filled with degassed buffer, equilibrated with 1, 1,2, and 1/4 atm NO (2,000, 1,000, and 500 μM, respectively), and –100 μM deoxy-CerHb was added. Bimolecular recombination was measured at 436 nm using the 300-ns dye laser for photolysis. The value of k'CO was taken from the slope of kobs versus [CO].

O₂ dissociation rate coefficients were determined by analyzing time courses for ligand replacement in a stopped-flow spectrometer. In these experiments, 5–10 μM CerHbO₂ samples in buffer containing various concentrations of free O₂ were mixed with buffer equilibrated with 1 atm CO. The O₂ dissociation rate coefficients were calculated from kₐCO = rₐCO / (1 + kₐCO [O₂] / [CO]), where rₐCO is the observed first order replacement rate coefficient, and kₐCO and kₐO₂ are the association rate coefficients for CO and O₂ binding, which were determined independently (29).

CO association time courses were measured either in stopped-flow rapid mixing experiments, in which the reduced deoxygenated protein was mixed with various concentrations of CO, or in laser photolysis experiments where rebinding was followed after complete photodissociation. Rate coefficients for CO dissociation were determined by analyzing the time courses, in which the heme-bound CO is displaced with high concentrations of NO (28, 29). Equilibrium coefficients for O₂ and CO binding were calculated from the ratio of the association and dissociation rate coefficients.

**Cryotransform Infrared Temperature Derivative Spectroscopy—**FTIR-TDS is an experimental protocol designed to measure thermally activated rate processes with distributed barriers (35). In the first step, the sample is photodissociated by a specific illumination protocol that selectively populates the desired intermediate state(s). Subsequently, FTIR transmission spectra are taken every kelvin while the temperature is increased linearly in time (typically at a rate 5 mK/s) over a controlled temperature interval. The FTIR and TDS analyses are calculated for successive temperatures. Frequently, the change in the spectral area of an infrared band that occurs during acquisition of two successive spectra can be taken as proportional to the change in the population of CO molecules contributing to the band. Population changes arise from ligand rebinding and ligand diffusion to and from different docking sites. The rate of the different rate process can be determined by thermal activation. The temperature ramp protocol ensures that they occur sequentially with respect to the height of the activation enthalpy barrier. For a simple two-state reaction, the temperature axis can be converted to an enthalpy axis, with the barrier height approximately proportional to the ramp temperature. The FTIR data are presented as contour plots of the absorbance change factor versus wave number and temperature axes, with solid (dotted) lines indicating increasing (decreasing) absorbance.

**Flash Photolysis at Cryogenic Temperature—**For low temperature flash photolysis experiments, dilute samples with a protein concentra-


Fig. 1. a, \( O_2 \) rebinding to wild-type CerHb, Thr-E11 \( \rightarrow \) Val CerHb, and wild-type sperm whale (sw) \( M_{b} \) at pH 7.0, 20 °C. The concentration of \( O_2 \) was 1.250 \( \mu \text{M} \) buffer equilibrated with 1 atm pure \( O_2 \). \( O_2 \) displacement from wild-type \( C. \text{lacteus} \) \( M_{b} \), Thr-E11 \( \rightarrow \) Val \( C. \text{lacteus} \) \( M_{b} \), and wild-type sperm whale \( M_{b} \) at pH 7.0, 20 °C is shown on short (b) and long (c) time scales.

Table I

| Protein                          | \( k_{o2}^{\prime} \) | \( k_{o2} \) | \( K_{o2} \) |
|----------------------------------|-----------------------|---|---|
| Native body wall CerHb           | 340 | 640 | 0.53 |
| Wild-type nerve CerHb            | 240 | 180 | 1.3 |
| Thr-E11 \( \rightarrow \) Val CerHb | 30  | 0.18 | 170 |
| Thr-E11 \( \rightarrow \) Ala CerHb | 41  | 0.26 | 160 |
| Tyr-B10 \( \rightarrow \) Ala CerHb | 120 | 440 | 0.3 |
| Tyr-B10 \( \rightarrow \) Phe CerHb | 140 | 160 | 0.3 |
| Gln-E7 \( \rightarrow \) His CerHb | 130 | 65  | 1.9 |
| Gln-E7 \( \rightarrow \) Leu CerHb | 200 | 96  | 2.0 |
| Wild-type sperm whale \( M_{b} \) | 17  | 15  | 1.1 |
| Ascaris Hb                       | 1.5 | 0.004 | 370 |

* These data were included because they are the only link to the native protein as it is obtained from the animal. All other CerHbs in the table were recombinant proteins.

\( \mu \text{M}^{-1} \text{s}^{-1} \phi \text{mol}^{-1} \text{s}^{-1} \)

Table II

| Protein                          | \( k_{o2}^{\prime} \) | \( k_{o2} \) | \( K_{o2} \) |
|----------------------------------|-----------------------|---|---|
| Native body wall CerHb           | 340 | 640 | 0.53 |
| Wild-type nerve CerHb            | 240 | 180 | 1.3 |
| Thr-E11 \( \rightarrow \) Val CerHb | 30  | 0.18 | 170 |
| Thr-E11 \( \rightarrow \) Ala CerHb | 41  | 0.26 | 160 |
| Tyr-B10 \( \rightarrow \) Ala CerHb | 120 | 440 | 0.3 |
| Tyr-B10 \( \rightarrow \) Phe CerHb | 140 | 160 | 0.3 |
| Gln-E7 \( \rightarrow \) His CerHb | 130 | 65  | 1.9 |
| Gln-E7 \( \rightarrow \) Leu CerHb | 200 | 96  | 2.0 |
| Wild-type sperm whale \( M_{b} \) | 17  | 15  | 1.1 |
| Ascaris Hb                       | 1.5 | 0.004 | 370 |

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\( \mu \text{M}^{-1} \text{s}^{-1} \phi \text{mol}^{-1} \text{s}^{-1} \)

RESULTS

O\(_{2}\) Binding to Wild-type and Distal Pocket Mutants of CerHb—In order to examine how CerHb is able to achieve an Mb-like \( O_2 \) affinity, we replaced Tyr-B10, Gln-E7, and Thr-E11 with apolar residues of similar size and surveyed the overall \( O_2 \) binding parameters of recombinant nerve CerHb. Typical time courses for \( O_2 \) binding and dissociation are shown in Fig. 1. The computed association, dissociation, and equilibrium coefficients for the series of single mutants are given in Table I. Rate coefficients for \( O_2 \) binding to body wall CerHb isolated from intact worms were determined as controls for comparison with the values for the recombinant nerve proteins. Samples of native nerve CerHb were not available.

Although nerve and body wall CerHbs have \( O_2 \) affinities similar to mammalian Ms, their association and dissociation rate coefficients are both 10-fold larger than those of muscle Ms (Table I). The value of \( k^{o2}_{o2} \) \( = \) 240 \( \mu \text{M}^{-1} \text{s}^{-1} \) for wild-type nerve CerHb indicates an easily accessible distal pocket and suggests that the rate-limiting step for \( O_2 \) binding is movement from the solvent into the protein interior. To confirm this conclusion, we measured the bimolecular rate of NO binding to reduced wild-type CerHb and observed \( k_{N2O} \) \( = \) 230 \( \mu \text{M}^{-1} \text{s}^{-1} \) (Scott et al., 36) have shown that \( k_{N2O} \) is equal to the bimolecular rate of ligand entry into all mutant Ms that have penta-coordinate deoxyxgenated heme groups. Thus, the equivalence of \( k_{N2O} \) and \( k_{o2}^{o2} \) indicates that \( O_2 \) binding to wild-type CerHb is diffusion controlled.

The rate coefficients for \( O_2 \) dissociation from wild-type nerve and native body wall CerHbs are also remarkably large (Table I) compared with most other \( O_2 \)-binding proteins containing polar groups in their distal pockets. Mutation of Gln-E7 in CerHb to either His or Leu causes only small changes (2-fold) in \( k_{o2}^{o2} \) (Fig. 1b) and a spectacular 1,000-fold decrease in the rate of \( O_2 \) dissociation (Fig. 1, b and c, and Table I). This single mutation converts CerHb from a protein with a modest \( O_2 \) affinity (\( P_{50} \) \( \approx \) 0.6 torr) typical of mammalian Ms and Hbs with \( O_2 \) storage/transport functions to an ultra-high-affinity Hb (\( P_{50} \) \( \approx \) 0.003 torr).

**Overall CO Binding to Wild-type and Thr-E11 \( \rightarrow \) Val CerHb at Ambient Temperatures**—Overall rate coefficients for CO binding to wild-type neural, native body wall and Thr-E11 \( \rightarrow \) Val CerHb are listed in Table II and compared with the corresponding parameters for CO binding to wild-type sperm whale Mb and native Ascaris Hb, which has Tyr-B10, Gln-E7, and

\( \mu \text{M}^{-1} \text{s}^{-1} \phi \text{mol}^{-1} \text{s}^{-1} \)

\( \mu \text{M}^{-1} \text{s}^{-1} \phi \text{mol}^{-1} \text{s}^{-1} \)}
Ile-E11 residues in the distal pocket. The CO association rate coefficients for CerHb are 100-fold higher than those for other animal Hbs and Msbs, indicating that the iron atom is highly reactive and accessible in the wild-type protein. The Thr-E11 to Val mutation causes −10- and −7-fold decreases in $k_{\text{CO}}$ and $k_{\text{CO}}/k_{\text{O2}}$, respectively (Table II). The net result is little change in CO affinity, which is in striking contrast to the −130-fold increase in $O_2$ affinity produced by the same mutation (Table I).

**Determination of the Crystal Structure of Thr-E11 → Val CerHbO$_2$**—In order to examine the biophysical mechanisms behind the dramatic effect of the Thr-E11 hydroxyl group on ligand binding, we determined the three-dimensional structure of Thr-E11 → Val CerHbO$_2$ using difference Fourier methods, and we compared it with that of the wild-type protein (22). The final model was refined to 1.7 Å resolution. The mutant structure contains 109 residues (plus an extra N-terminal Met residue), 92 water molecules, one bound O$_2$ molecule, and one sulfate anion ($R$-factor = 16.7%, $R$-free = 19.5%), with ideal stereochemical parameters (37) (Table III).

Optimal superposition of the Thr-E11 → Val mutant and the wild-type CerHbO$_2$ structures yielded an r.m.s.d. value of 0.12 Å (calculated for 109 C-α atom pairs). Thus, the two molecules are virtually structurally indistinguishable, despite their remarkably different $O_2$ binding properties. There are no significant changes (≤0.05 Å) in the Fe–O$_2$ and Fe–N-e (His-F8) coordination bond distances. The staggered azimuthal orientation of the proximal His-F8 side chain relative to the heme pyrrole N atoms is completely conserved, presumably because the imidazole ring makes energetically equivalent van der Waals contacts with residue Phe-G5 and forms a hydrogen bond to the Leu-F8 carbonyl O atom in both the wild-type and mutant proteins.

The only significant structural differences between the wild-type and mutant proteins are movements of the Tyr-B10 and Val-E11 side chains away from each other and a slight “inward” movement of the heme-bound O$_2$ molecule in the Thr-E11 → Val mutant (Fig. 2). In wild-type CerHbO$_2$, the distance between the O-η atom of the Tyr-B10 side chain and the O-γ1 atom of the Thr-E11 hydroxyl group is only 2.59 Å, implying that a strong hydrogen bond is “holding” the Tyr-B10 side chain near the Thr-E11 helical position (Table IV). The Thr-E11 → Val mutation results in rotations of the Tyr-B10 side chain about the Cα-Cβ and Cβ-Cγ bonds, which shift the O-η atom of Tyr-B10 roughly 0.7 Å away from the C-γ2 atom of → Val-E11 and toward the heme-bound ligand (Fig. 2 and Table IV). The close approach of the Tyr side chain to the heme-bound ligand causes a slight bending of the Fe-O$_2$ complex.

The N-ε atom of the Gln-E7 side chain in wild-type CerHb is also close to the heme-bound ligand (Table IV). There appears to be a moderately strong hydrogen bond with the O(2) ligand atom and a much weaker interaction with the O(1) atom, based on N-ε → O distances of 2.61 and 3.35 Å, respectively (Table IV). In the Thr-E11 → Val CerHbO$_2$ mutant, the heme-bound ligand moves away from the Gln-E7 side chain, increasing the N-ε → O (2) distance to 2.80 Å. However, the Tyr-B10 hydroxyl O-ε atom moves to within ~3 Å of the amide side chain and may be competing with the ligand for the N-ε proton.

In wild-type CerHbO$_2$, the Thr-E11 O-γ1 atom is 3.12 Å away from the main chain O atom of the Gln-E7, the amino acid that is one turn toward the N-terminal of the E-helix (Table IV). This observation suggests strongly that the Thr-E11 hydroxyl group is donating a proton to the carbonyl O atom. In the Thr-E11 → Val CerHbO$_2$ mutant, the apolar mutant side chain rotates in the opposite direction, downward and toward the heme, causing the distance between the Gln-E7 carbonyl O
The hydrogen bond between the Tyr-B10 hydroxyl group and the Gln-E7 carbonyl defines the direction of the electrostatic interaction with the Tyr-B10 side chain (see arrows in Fig. 2). The nonbonded electrons of the Tyr-E11 O-y1 atom are pointing toward the Tyr-B10 side chain and accept a proton from the phenolic O-atom. This hydrogen bond appears to be strong since the oxygen atoms are 2.59 A (Table IV). Donation of the phenolic proton to the Thr-E11 hydroxyl group directs the nonbonded electrons of the O-atom of Tyr-B10 toward the heme-bound ligand, which, in the case of heme-bound O2, also has a partial negative charge. This electrostatic repulsion provides a mechanistic explanation for the much lower O2 affinity and much higher O2 dissociation rate coefficients in the wild-type protein as compared with the Thr-E11 Val mutant, where the phenolic proton of Tyr-B10 can be donated to the heme-bound ligand and stabilize it.

**FTIR Absorbance Difference Spectra at 3 K**—Extensive studies on wild-type sperm whale Mb and a large number of single mutants have shown that ligands sample several transient docking sites on their way into and out of the protein (36, 43–50). At cryogenic temperatures, the ligands can be trapped selectively at these intermediate sites. The rates and the extent of ligand migration among these docking sites at cryogenic temperatures provide clues about the physiological ligand binding process. To survey the existence of well defined transient docking sites in CerHb, FTIR absorbance difference spectra of wild-type and Thr-E11 Val CerHbCO were recorded after a 1-s illumination at 3 K to disrupt the bond between the heme iron and CO (Fig. 4). These spectra consist of a contribution in the spectral region of heme-bound CO that represents the fraction of iron-ligand complexes or A states that can be

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**TABLE IV**

| Atom pair                          | Distance, wild-type | Distance Val-E11 mutant |
|------------------------------------|---------------------|-------------------------|
| Tyr-B10, O-→ O(1) (ligand)         | 2.58                | 2.51                    |
| Tyr-B10, O-→ O(2) (ligand)         | 3.16                | 2.86                    |
| Tyr-B10, O-→ O(1) (ligand)         | 2.59                |                         |
| Tyr-B10, O-→ Val-E11, C-γ2         | 3.30                |                         |
| Gln-E7, N-→ O(2) (ligand)          | 2.61                | 2.80                    |
| Gln-E7, N-→ O(1) (ligand)          | 3.35                | 3.63                    |
| Gln-E7, N-→ Tyr-B10, O-            | 3.29                | 2.99                    |
| Thr-E11, O-→ Gln-E7, O (carbonyl)  | 3.12                |                         |
| Val-E11, C-γ2→ Gln-E7, O (carbonyl)| 3.45                |                         |
| Thr-E11, O-→ O(1) (ligand)         | 4.43                |                         |
| Val-E11, C-γ2→ O(1) (ligand)       | 4.36                |                         |
| Thr-E11, O-→ O(2) (ligand)         | 4.42                |                         |
| Val-E11, C-γ2→ O(2) (ligand)       | 4.31                |                         |
| Thr-E11, O-→ Gln-E7, N-            | 3.51                |                         |
| Val-E11, C-γ2→ Gln-E7, N-          | 3.62                |                         |

atom and the C-γ2 atom to increase by ~0.3 A (Table IV and Fig. 2). The hydrogen bond between the Thr-E11 hydroxyl group and the Gln-E7 carbonyl defines the direction of the electrostatic interaction with the Tyr-B10 side chain (see arrows in Fig. 2). The nonbonded electrons of the Thr-E11 O-atom are pointing toward the Tyr-B10 side chain and accept a proton from the phenolic O-atom. This hydrogen bond appears to be strong since the oxygen atoms are 2.59 A (Table IV). Donation of the phenolic proton to the Thr-E11 hydroxyl group directs the nonbonded electrons of the O-atom of Tyr-B10 toward the heme-bound ligand, which, in the case of heme-bound O2, also has a partial negative charge. This electrostatic repulsion provides a mechanistic explanation for the much lower O2 affinity and much higher O2 dissociation rate coefficient in the wild-type protein as compared with the Thr-E11 Val mutant, where the phenolic proton of Tyr-B10 can be donated to the heme-bound ligand and stabilize it.

**FTIR Absorbance Difference Spectra at 3 K**—Our interpretations of the active site structures of wild-type and Thr-E11 Val CerHbO2 predict a pronounced difference in the electric field experienced by the heme-bound ligand because in the mutant, the positive charge of the phenolic hydrogen points toward the ligand instead of the negative charge of the Tyr-B10 phenolic oxygen lone pair. Several researchers have shown that there is linear correlation between the CO stretching frequency νCO and the electrostatic field adjacent to the heme-bound CO (38–42). Electric fields provided by strong or multiple hydrogen bond donors correlate with νCO peaks in the 1910–1930 cm⁻¹ region. Moderate hydrogen bonding interactions yield peaks between 1940 and 1950 cm⁻¹. Neutral (apolar) environments and negative partial charges in the vicinity of the CO oxygen give rise to νCO bands from 1960 to 1970 and 1970 to 1990 cm⁻¹, respectively.

At 298 K, the FTIR spectrum of wild-type CerHbCO at pH 7.0 shows a single peak of heme-bound CO at 1979 cm⁻¹, which is indicative of a negative charge close to the bound ligand (Fig. 3). This result supports our interpretation of the direction of the electrostatic interactions between the side chains of Tyr-B10 and Thr-E11 and the main chain carbonyl of Gln-E7 in wild-type CerHb. The nonbonded electrons of the Tyr-B10 hydroxyl group are pointed toward the heme-bound CO, stabilizing the Fe-C≡O tautomer. Replacing Thr-E11 with either Ala or Val causes a dramatic 40 cm⁻¹ decrease in νCO to 1930–1940 cm⁻¹, indicating a reversal of the electric field (Fig. 3). This shift correlates with the movement of the Tyr-B10 side chain and rotation of the phenolic hydrogen toward the heme-bound ligand, enhancing the fraction of Fe= C=O tautomer.
that either there is only one major photoproduct with a single CO orientation or the Stark effects are too small to cause differences in the CO stretch frequency of the different CO conformers.

Cooling under continuous illumination enables photodissociated ligands to populate additional transient docking sites with higher recombination barriers (51). In sperm whale Mb, ligands have been recovered in internal hydrophobic pockets called xenon cavities after their ability to bind large noble gases (43–45, 49, 50, 52, 53). In CerHbCO, ligands can also migrate away from the initial docking site as is evident from the pronounced changes in the photoproduct spectrum after extended illumination (Fig. 4). The major photoproduct band is shifted from 2125 to 2122 cm⁻¹, and the minor band at 2134 cm⁻¹ is significantly enhanced.

Replacement of Thr-E11 with Val gives rise to a dominant νCO band at 1938 cm⁻¹ and a minor band at 1955 cm⁻¹ for heme-bound CO (Fig. 4). In the spectral region of photolyzed CO, two major bands are observed at 2123 and 2142 cm⁻¹, and two minor bands at intermediate positions (2131 and 2134 cm⁻¹) appear as a broad feature. The existence of four peaks implies that ligands populate more than one intermediate site even after only brief illumination at 3 K. Extended illumination markedly enhances the absorbance of the bands centered at 2131 and 2134 cm⁻¹, but small fractions of the other two bands remain. Thus, the features in the 2131–2134 cm⁻¹ region appear to represent ligands at sites that are further away from the iron atom or have a higher energy barrier against recombination.

TDS on Wild-type CerHbCO—In TDS experiments, populations of the photodissociated ligands can be sorted according to the temperature at which they rebind to the iron atom. This temperature is a measure of the enthalpy barrier to geminate recombination. The method also helps to establish the number of intermediate docking sites and their order of interconversion (35).

In a first experiment, the absorbance changes in the bands of heme-bound CO were monitored as a function of temperature after wild-type CerHbCO was photolyzed at 3 K with a brief 1-s illumination (Fig. 5a). CO rebinding started immediately, even at 3 K, as seen from the prominent contours centered at ~1982 cm⁻¹. This result demonstrates unambiguously that the majority of the photodissociated ligands have very low enthalpy barriers against recombination at the iron. Less intense contours at ~1938 cm⁻¹ arise from the 13C16O isotope re-binding in the same heme-bound conformation.

The absorbance changes in the photoproduct bands are shown in Fig. 5b. The decrease in the major broad feature centered at 2125 cm⁻¹ is associated with ligand re-binding to the heme iron from the initial docking site B and correlates directly with the absorbance increase at ~5 K in the dominant A state band (Fig. 5a). It can be resolved into two narrower bands with peaks at 2122 and 2126 cm⁻¹ (Fig. 5b). A small solid contour at 2134 cm⁻¹ and ~20 K indicates thermally induced ligand migration to an additional site, which is designated state C. Ligands rebind from this state at significantly higher temperatures, ~50 K, than from the initial B states. This process is seen as the peak in the photoproduction map at ~2134 cm⁻¹.

This pattern is quite different from that in sperm whale Mb (44), where recombination peaks at ~50 K for the A₁ (νCO = 1945 cm⁻¹) and at ~80 K for the A₂ conformer (νCO = 1933 cm⁻¹). Moreover, there are two spectroscopically well separated B state bands, B₁ at 2131 cm⁻¹ and B₂ at 2119 cm⁻¹ (Table II). Conversion of B₂ to B₁ occurs in the 10–30 K temperature range. Re-binding in A₂ occurs exclusively from B₁ at 50 K.
A second TDS experiment was performed after wild-type CerHbCO was slowly cooled from 160 to 3 K under constant illumination (Fig. 6, a and b). At 160 K, all heme-bound CO molecules are photolyzed. At these higher temperatures, ligands may rebind but will be photolyzed again. Because the temperature is ramped down, rebinding will be slowed continuously. Ligands will eventually have insufficient thermal energy to overcome the barriers against recombination and become trapped in more remote sites, each of which will have a characteristic distribution of enthalpy barriers \( g(H_{BA}) \).

The resulting TDS maps of wild-type CerHb differ markedly from the ones generated after brief illumination at 3 K. Instead of re-binding readily at \(-5\) K, most of the dissociated CO molecules rebind at much higher temperature, \(-80\) K. Small subpopulations still show low barriers with recombination peaks at \(-5\) and \(-40\) K. In the photoproduct map (Fig. 6b), there are no features at low temperatures corresponding to rebinding from the initial docking site. Instead, absorbance decreases in the region associated with state C (2131–2134 cm\(^{-1}\)) and peaks at \(-40\) K. Solid contours in this same temperature range imply either (i) that the photolyzed ligands do not recombine directly from intermediate state C, but migrate to yet another intermediate site D where they finally rebind at \(-80\) K, or (ii) ligand reorientation at site D. Recombination from state D is indicated by the feature centered at 2123 cm\(^{-1}\) and 80 K.

After prolonged illumination of sperm whale MbCO, many key features of the 1-s illumination photoproduct map are retained (44). "New" solid and dotted contours indicate ligand migration into and recombination from the Xe-4 and Xe-1 pockets (states C and D), but the extent of the migration toward more remote sites is much less than that seen for CerHb.

**Temperature Derivative Spectroscopy on Thr-E11 \(-\) Val CerHb**—The A state contour maps of the Thr-E11 \(-\) Val CerHbCO mutant after both short and prolonged illumination are much more complex than those of the wild-type protein (Figs. 5, c and d, and Fig. 6, c and d). The mutant shows a very broad band of heme-bound CO centered at 1938 cm\(^{-1}\) and minor features in the 1950–1960 cm\(^{-1}\) region, indicating multiple A state conformations. After a 1-s illumination, rebinding to form the dominant state is maximal at \(-30\) K. In the minor A states (1955 and 1968 cm\(^{-1}\)) recombination occurs maximally at 70 and 5 K, respectively.

The spectral features in the photoproduct map of the Thr-E11 \(-\) Val mutant are much more dispersed (2115–2145 cm\(^{-1}\)) than those for wild-type CerHb. The large solid contours at 2134 cm\(^{-1}\) and \(-20\) K indicate significant thermally induced migration from the two initial photoproducts, with peaks at 2123 and 2145 cm\(^{-1}\), to a new state, most likely the same state C seen in the wild-type protein (Fig. 5b). Rebinding from the secondary site C is seen as dotted contours centered at 2134 cm\(^{-1}\) and peaking at \(-50\) K (Fig. 6d).

The A state contour plot of the Thr-E11 \(-\) Val CerHb mutant after extended illumination shows that the dominant A state subpopulation still forms maximally at \(-40\) K (Fig. 6c). Only a few ligands have migrated to more remote sites. Rebinding from these sites occurs at markedly higher temperatures (\(-90\) and \(-150\) K, Fig. 6c). The photoproduct map is now dominated by contours at 2134 cm\(^{-1}\), representing rebinding from state C that occurs maximally at \(-50\) K (Fig. 6d). However, the exchange seen in the 1-s illumination experiment for the B to C state transition is absent (Fig. 5d and Fig. 6d).

**Flash Photolysis at Cryogenic Temperatures**—To investigate the enthalpy barriers against ligand rebinding and migration
more quantitatively, laser photolysis experiments were performed at cryogenic temperatures, and absorbance changes for geminate recombination in wild-type and Thr-E11 → Val CerHb were monitored at 436 nm (Fig. 7). The time courses between 40 and 180 K were fitted with a two-state model that invokes a static distribution of activation enthalpy barriers, g(H), between bound and photoprotein state. The fraction of heme groups that is still unligated at time t after the flash, N(t), is given by Equation 1.

\[ N(t) = \int g(H) \exp(b(H,T)t) \, dH \]  

(Eq. 1)

The measured absorbance change, ΔA(t), is taken to be proportional to N(t). For a thermally activated barrier crossing, the dependence of the rebinding rate coefficient k(H,T) on temperature T is given by the transition state expression shown in Equation 2.

\[ k = A \frac{T}{T_0} \exp \left( -\frac{H}{kT} \right) \]  

(Eq. 2)

with pre-exponential constant A, reference temperature \( T_0 \) set to 100 K, and the universal gas constant \( R \). In the calculations, the \( \Gamma \) distribution (Equation 3) was chosen as a model function for g(H) (54, 55).

\[ \Gamma(H) = (H - H_{\text{min}})^{\alpha(H_{\text{max}} - H_{\text{min}})} \exp(-\alpha(H - H_{\text{min}})) \]  

(Eq. 3)

For wild-type CerHbCO, global nonlinear least squares fitting of the experimental data yielded a pre-exponential \( A = 10^{15} \text{s}^{-1} \), with the g(H) distribution peaking at 2.2 ± 0.2 kJ/mol (\( \alpha = 1.15 \text{ mol/kJ}; H_{\text{min}} = 0.5 \text{ kJ/mol} \), see Table II). The data, however, deviate from the calculated kinetic traces starting at 60 K. At times longer than 100 ms, recombination is slower than predicted. Based on the TDS data, we propose that a fraction of the photodissociated ligands migrates to state C during the initial recombination phase and that the slower geminate phase represents ligand recombination from this more remote site. By contrast, the experimental traces for the Thr-E11 → Val CerHbCO mutant in the 60–180 K range (Fig. 7b) can be fitted with a single g(H) distribution, with parameters \( A = 10^{8.5} \text{s}^{-1} \) and \( H_{\text{peak}} = 5.77 \pm 0.2 \text{ kJ/mol} \) (\( \alpha = 0.82 \text{ mol/kJ}; H_{\text{min}} = 0.5 \text{ kJ/mol} \), see Table II). Only the experimental data at 40 K and below deviate significantly from the fit. At these very low temperatures, recombination is faster than predicted, due to quantum-mechanical tunneling of the CO (55, 56). The markedly increased enthalpic barrier in the Thr-E11 → Val mutant compared with that in the wild-type CerHb implies that rebinding probably occurs exclusively from intermediate site C.

**DISCUSSION**

*Electrostatic Regulation of O2 Affinity*—Our structural interpretation of how Thr-E11 decreases the O2 affinity in wild-type CerHb is shown in Fig. 2. The Thr-E11 hydroxyl group donates its proton to the main chain carbonyl O atom of Gln-E7. A similar interaction is observed in the Val-E11 to Thr mutant of pig Mb (57), where the Thr-E11 side chain is rotated about the Cα–Cβ bond, upward and away from the heme, bringing the O-y1 atom of Thr-E11 0.5 Å closer to the His-E7 carbonyl O atom. In fact, this hydrogen bonding pattern is seen for most Thr side chains present in α-helices, particularly when the face containing the hydroxyl group is located in an apolar environment (i.e. in membranes or facing the interior of a protein (58)).

The nonbonded electrons of the Thr-E11 O-y1 atom redirect the phenolic hydrogen of Tyr-B10 away from the bound ligand to form a strong hydrogen bond between the two amino acid side chains. This interaction “holds” Tyr-B10 next to the E-helix. Instead of donating a favorable hydrogen bond to the heme-bound ligand, the nonbonded electron pairs of the Tyr-B10 O-y atom point directly toward the ligand. The resulting electric field destabilizes the heme-bound O2 by electrostatic repulsion, accounting for the large dissociation rate coefficient and moderate \( K_{O2} \) value, despite the favorable proximal geometry and the hydrogen bonding interaction with the amide side chain of Gln-E7. As shown in Fig. 2 and Table IV, the Thr-E11 side chain in wild-type CerHbO2 cannot interact directly with the heme-bound ligand (distance ≥4.3 Å) or strongly with the Gln-E7 side chain (distance ≤3.4 Å). These distances do not change in the Thr-E11 → Val mutant. This geometry is significantly different from that in mammalian Mbs, where both the C-y2 atom of the naturally occurring Val-E11 side chain and the O-y1 atom in Thr-E11 mutants are within 3 Å of the heme-bound ligand and interact directly and unfavorably with it (57, 59).

The dramatic functional effects caused by replacing Thr-E11 with Val confirm the proposed mechanism for regulating O2 affinity. This isosteric mutation causes a 0.7-Å movement of the Tyr-B10 hydroxyl group away from the Val-E11 side chain and a 0.3-Å movement of the C-y2 atom of Val-E11 away from the main chain carbonyl O atom of Gln-E7 (Fig. 2 and Table IV). Both movements reflect disruption of the hydrogen bonding network that is present in the wild-type CerHb due to the Thr-E11 hydroxyl group. In the Thr-E11 → Val CerHbO2 mutant, the Tyr-B10 group is “free” to donate a strong hydrogen bond to bound ligands. This favorable interaction is manifested
by a dramatic increase in $O_2$ affinity and a decrease in $k_{\text{on}}$. The conclusion that the Thr-E11 to Val mutation causes a reversal of the electric field at the ligand-binding site is strongly supported by the FTIR spectra of the corresponding CerHbCO complexes (Figs. 3 and 4 and Table II). At both ambient and low temperatures, heme-bound CO in wild-type CerHb shows a narrow band centered at $-1980 \text{ cm}^{-1}$, a frequency that is only seen in model heme compounds and mutant sperm whale Msbs that have negative partial charges adjacent to the heme-bound ligand (38–42). The Thr-E11 $\rightarrow$ Val mutation causes a shift of the $v_{\text{CO}}$ band to $1998 \text{ cm}^{-1}$, a frequency characteristic of model compounds and Msbs with strong hydrogen bonds between the heme-bound ligand and surrounding amino acid side chains, usually His-E7, Gln-E7, Tyr-B10, and/or Asn-E11 (38–42).

**Thr-E11 and Ligand Selectivity**—The effects of the Thr-E11 to Val mutation on the functional properties of CerHb provide dramatic confirmation of the theory that distal electrostatic interactions, and not steric hindrance, govern ligand selectivity in heme proteins. Despite the change in the electrostatic field, the Thr-E11 to Val mutation causes little net change in the overall affinity for CO because the FeCO complex is inherently neutral (Table II). In contrast, $K_{\text{d}}$ increases 130-fold due to the change from the unfavorable electrostatic repulsion between the bound ligand and the nonbonded electrons of the Tyr-B10 O-$\eta$ atom to the formation of a strong hydrogen bond between the phenolic hydroxyl group and the highly polar FeO$_2$ complex. Thus, the Thr-E11 to Val mutation changes CerHb from a protein that favors CO binding by a factor of ~400 to a mutant protein that has roughly equal affinity for CO and O$_2$ ($M$ values in Table II). These marked changes occur with little or no alteration of protein structure and heme-ligand coordination geometry (Fig. 2). Similar selective enhancement of the $O_2$ affinity is seen when hydrogen bonding between Tyr-B10 and heme-bound $O_2$ occurs naturally (Table II, very low $M$ value for Ascaris Hb).

**Regulation of the Overall Rates of Ligand Binding**—The formation of strong hydrogen bonds with heme-bound $O_2$ requires side chain atoms of adjacent polar amino acids to be within 2.5–3.0 Å of the sixth coordination position of the heme iron. This close proximity often reduces the rate coefficient for ligand association because access to the iron atom is hindered in the dissociated “deoxy” state by the polar side chains, which either move closer to the iron atom or bind solvent water molecules. Thus, in mammalian Msbs, mutation of the distal His-E7 to apolar amino acids results in 5–to 60-fold increases in the association rate coefficients for $O_2$ and CO binding, even though the $O_2$ affinity decreases markedly (60). In wild-type CerHb, the opposite situation occurs. The Tyr-B10 side chain is held next to the E-helix by hydrogen bonding to Thr-E11, preventing the aromatic side chain from swinging toward the heme iron, accounting in part for the large absolute values of $k_{\text{on}}$ and $k_{\text{CO}}$. When Thr-E11 is replaced by Val, however, the Tyr-B10 side chain moves freely toward the binding site and the phenolic hydroxyl group inhibits access to the heme iron atom in the unliganded state. This movement gives rise to the 10-fold decreases in both $k_{\text{on}}$ and $k_{\text{CO}}$ and the marked increase in the enthalpic barrier against geminate recombination (Tables I and II). The large absolute values of $k_{\text{on}}$ and $k_{\text{CO}}$ of wild-type CerHb are also the result of the staggered orientation of the proximal imidazole base, which reduces the barrier to in-plane movement of the iron atom, and the ease of ligand entry into the protein, presumably through the apolar interior tunnel.

**Structural Interpretations of the Photoproduct States and Geminate Recombination**—As shown in Figs. 3–7, altering the hydrogen bonding network between Tyr-B10, Thr-E11, and the carbonyl O atom of Gln-E7 has profound effects on the kinetic and spectral properties of photodissociated CO trapped in the protein. In wild-type Mb and most mutants that retain His-E7, the initial photoproduct generated by short illumination at 3 K exhibits two IR bands. Lim et al. (61) have investigated the nature of ligand motion within Mb by measuring femtosecond time-resolved infrared spectra of photolyzed CO. They observed two trajectories for the CO ligands into the initial docking site B, which can be distinguished kinetically. The trajectory that leads to the faster rebinding B state was assigned to CO molecules that translate directly into the docking site with the carbon atom still pointing toward the heme iron. The other trajectory involves rotation of the ligand molecule into the initial docking site with the O atom pointing back toward the iron, explaining why it cannot rebind without first rotating into the other reactive B state orientation. These two conformers are readily distinguished spectroscopically, due primarily to interactions with the N-$\sigma$ proton of His-E7 that cause a Stark splitting into two photoproduct bands. This electrostatic interaction enhances the triple bond character of the $B_1$ substate with the C atom pointing toward the iron and His-E7, giving rise to the $v_{\text{CO}}$ band at 2131 cm$^{-1}$. In contrast, interaction with His-E7 decreases the bond order of CO in the $B_2$ substate where the O atom is pointing back toward the iron, resulting in the $v_{\text{CO}}$ band at 2119 cm$^{-1}$ (43). When the distal His-E7 in Mb is replaced with Val or Leu, the splitting is markedly reduced or gone, and the barriers to recombination are reduced significantly.

In wild-type CerHb, the Tyr-B10 side chain is held away from the iron and the photodissociated ligands. As a result, there is little Stark splitting of the bands for the two CO orientations in the initial docking site B and little or no steric barrier against rebinding. In the TDS experiments after 1-s illumination, the $T_{\text{max}}$ value for rebinding to wild-type CerHb is very low, $-5$ K compared with $-50$ K for wild-type sperm whale Mb, and the peak value of the enthalpy barrier against geminate rebinding from site B is only ~2 kJ/mol compared with $-10$ kJ/mol for wild-type sperm whale Mb (Table II). Prolonged illumination (“pumping”) of CerHbCO is required to trap photodissociated CO molecules at more remote sites C and D because rebinding from the initial state B is so fast (Fig. 6b).

However, when Thr-E11 is replaced by Val, the Tyr-B10 side chain rotates back toward the iron, interacts with the photodissociated CO, causes marked splitting of the B state peaks (Fig. 4), and creates a large steric barrier to rebinding. As a consequence, the ligands that are initially trapped at site B will not rebind as readily to the heme iron, which allows them to escape to the more remote site C. This migration is indicated by the prominent positive peak in the photoproduct map of the mutant (Fig. 5d). Very similar features have been observed for Mb triple mutant YQR (Leu-B10 $\rightarrow$ Tyr/His-E7 $\rightarrow$ Gln/Tyr-E7 $\rightarrow$ Arg) (62). The photoproduct map of Thr-E11 $\rightarrow$ Val CerHb after prolonged illumination looks deceptively simple compared with the one of the wild-type CerHb (Fig. 6, $b$ versus $d$). There is a single broad feature centered at $-50$ K and 2134 cm$^{-1}$ indicating CO rebinding exclusively from the C site. This assignment is supported by the flash photolysis experiments at cryogenic temperatures. However, the A state map indicates additional rebinding at higher temperatures (i.e. the peak at 150 K, 1938 cm$^{-1}$) (Fig. 6c), presumably from ligands dispersed within the apolar channel.

Regardless of the exact structural interpretation, the prolonged illumination experiments show that ligands are trapped more readily at remote sites in both wild-type and mutant CerHb than in sperm whale Mb, despite the higher barrier.
recombination against recombination in the mammalian protein. Thus, the remote sites in the apolar channel in CerHb almost certainly play a key role in facilitating ligand capture and entry into the distal pocket. More mutation work is needed to define the exact role of the channel and to establish a detailed kinetic mechanism for ligand association in CerHb.

**Conclusions**—Although a mini-Cerb, CerHb is part of a series of naturally occurring globins with Gln and Tyr at the E7 and B10 positions. These proteins include bacterial Hbs, flavohemoglobin, and invertebrate Hbs, with the most well studied examples being *Vitreoscilla* Hb (63–66), *E. coli* (HMP) flavohemoglobin (67–72), and *Ascaris* Hb (73–75). The functions of these proteins are still controversial and may involve O2 sensing, NO dioxygenation, O2 scavenging, and peroxidase activity. Most researchers no longer believe that these hemoglobins serve as O2 storage proteins or transporters. All of them appear to have high O2 affinities and low O2 dissociation rate coefficients, when these parameters are measured directly (65, 67, 76, 77). Indicating that this protein almost certainly functions as an O2 scavenger. In contrast, the nerve and body wall CerHbs have moderate Mb-like O2 affinities, exhibit high O2 binding affinity, and display a low O2 dissociation rate coefficient.

Although many aspects of the active site of *Ascaris* and *C. lacteus* Hbs are structurally very similar. Both proteins show a staggered orientation of the porphyrin ring. This proximal geometry is characteristic of a highly reactive iron atom. Short distances between the side chains of Gln-E7 and Tyr-B10 and the heme-bound ligand indicate strong electrostatic interactions (24, 73). The key presence is the presence of the polar Thr-E11 side chain in the active site of CerHb instead of the apolar Ile-E11 side chain in *Ascaris* Hb. The mutagenesis results show that this single amino acid replacement is sufficient to explain the remarkably different physiological properties of the two Hbs. The single Thr-E11 → Val point mutation can convert CerHb from an O2 storage protein to an O2 scavenger analogous to *Ascaris* Hb and vice versa.

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Thr-E11 regulates O2 affinity in Cerebratulus lacteus mini-hemoglobin.

Alessandra Pesce, Marco Nardini, Paolo Ascenzi, Eva Geuens, Sylvia Dewilde, Luc Moens, Martino Bolognesi, Austen F. Riggs, Angela Hale, Pengchi Deng, G. Ulrich Nienhaus, John S. Olson, and Karin Nienhaus

Page 33665, Table II: The headings of the first three columns were incorrect. A corrected table is shown below:

| Protein                      | k_1^CO | k_2^CO | K_1^CO | M (K_1^CO/K_2^CO) | K_0^CO | K_0^CO | A | H_{geminate} |
|------------------------------|--------|--------|--------|------------------|--------|--------|----|--------------|
| Wild-type nerve CerHb        | 28     | 0.048  | 580    | 450              | 1962   | 2125   | 320 | 2.2^a        |
| Thr-E11 → Val CerHb          | 3.0    | 0.0070 | 430    | 2.5              | 1931   | 2123.2142 | 80 | 5.8^a        |
| Wild-type sperm whale Mb     | 0.53   | 0.019  | 27     | 0.03             | 1927,1945 | 2119,2131 | 630^a | 9.7^h,d      |
| Native Ascaris Hb^e           | 0.21   | 0.018  | 12     | 0.03             | 1910,1942 | ND      | ND | ND           |

* Recombination from site B.
^ ND, not determined.
^ Recombination from site C.
^ Data taken from Ref. 78.
^ Data from Refs. 25, 76, 79, and 80.

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Regulated production of a peroxisome proliferator-activated receptor-γ ligand during an early phase of adipocyte differentiation in 3T3-L1 adipocytes.

Iphigenia Tzameli, Hui Fang, Mario Ollero, Hang Shi, Jonathan K. Hamm, Paul Kievit, Anthony N. Hollenberg, and Jeffrey S. Flier

Page 36096, Fig. 3B: The label below the figure should read “–MIX br-cAMP Forsk.”

Page 36097, Fig. 4 legend: The second sentence should read: “The control medium (C3X) contained no DIM and was similarly treated.”

Page 36098, in the paragraph labeled “Specificity of the Ligand Activity”: The second sentence should read “The receptors tested included the PPARγ heterodimeric partner retinoic X receptor-α (RXRα), PPARα, estrogen receptor-α, and thyroid receptor-β.”

Page 36099, Fig. 7B legend, line 7: The sentence should read “B, ^35S-labeled TIF2 was incubated with bacterially expressed GST-PPARγ fusion protein in the presence of solvent (−), 1 µM rosiglitazone (Rosi), C100×, or CM100×, O/N.”

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