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Formation of two hydrogen bonds from the globin to the heme-linked oxygen molecule in *Ascaris* hemoglobin

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ABSTRACT **We have tried to find out why *Ascaris* hemoglobin has such an exceptionally high oxygen affinity ($P_{50} \approx$ 0.004 mmHg; 1 mmHg = 133 Pa). Following Kloek *et al.*, we have synthesized the N-terminal globin domain of *Ascaris* hemoglobin in *Escherichia coli* [Kloek, A. P., Yang, J., Mathews, F. S. & Goldberg, D. (1993) *J. Biol. Chem.* 268, 17669–17671]. Like Kloek *et al.*, we found its oxygen affinity to be as high as that of native *Ascaris* hemoglobin. We thought that this high affinity might be due to the heme-bound oxygen molecule being stabilized by two hydrogen bonds from the globin instead of the usual one. *Ascaris* hemoglobin has a distal glutamine instead of the more usual histidine as one of the potential hydrogen bond donors. In addition, it contains a tyrosine at position 10 of B helix (B10) in place of the leucine generally found there in vertebrate myoglobins and hemoglobins. Following the discovery of Carver *et al.* that sperm whale myoglobin with the replacement of leucine B10 by phenylalanine has a raised oxygen affinity, we have replaced tyrosine B10 in the N-terminal domain of *Ascaris* hemoglobin by either leucine or phenylalanine [Carver, T. E., Brantley, R. E., Jr., Singleton, E. W., Arduini, R. M., Quillin, H. L., Phillips, G. N., Jr., & Olson, J. S. (1992) *J. Biol. Chem.* 267, 14443–14450]. Either of these replacements lowered the oxygen affinity about 100-fold, to the same level of that of human α-globin chains. These results are consistent with a hydrogen bond linking the tyrosine hydroxyl to the heme-linked oxygen, with a bond energy of 2.7 kcal/mol.**

Ascaris is a parasitic nematode which lives in the intestines of animals where oxygen is scarce. It has a perienteric hemoglobin with an oxygen affinity about 100 times higher than that of vertebrate hemoglobins or of any iron-porphyrin synthesized so far (P_{50} = 0.004 mmHg) (1, 2). Davenport (2) showed that this high affinity was due to an extremely slow dissociation (off rate, k_{off} 0.0041/s^{-1}), whereas the association rate (on rate, k_{on}) was normal. By contrast, the affinity for carbon monoxide is similar to that of vertebrate hemoglobins. Synthetic chemistry has indicated no way of strengthening the Fe^{2+}—O_2 bond by the equivalent of nearly 3 kcal/mol.

The Fe^{2+}—O_2 bond is polar with partial Fe^{3+}—O_2^- character, whereas the Fe^{2+}—CO bond is nonpolar. In consequence, the iron-bound oxygen, but not the carbon monoxide, accepts a hydrogen bond from the distal histidine in myoglobin and in the α subunit of human hemoglobin (in the β subunit Fe and O_2 are too far apart) (3). In *Ascaris* hemoglobin the slow off rate of oxygen, in contrast to the normal off rate of carbon monoxide, suggested that the bound oxygen might be stabilized by an additional hydrogen bond from the globin.

The amino acid sequence of *Ascaris* hemoglobin showed that glutamine takes the place of the distal histidine (4, 5). This could serve equally well as a hydrogen bond donor to the bound oxygen, but that bond would be no stronger than the bond from histidine. Initially, the sequence gave no clue to the possible nature of a second hydrogen bond donor.

Such a clue did come from the observation of Olson and collaborators (6) that the replacement of leucine B10 (residue 29 of the polypeptide) in sperm whale myoglobin by phenylalanine raised its oxygen affinity 10-fold, because a hydrogen atom in the benzene ring of the phenylalanine made a favorable contact with the bound oxygen (6). In *Ascaris* hemoglobin this position is occupied by tyrosine. However, the myoglobin mutant B10 leucine → tyrosine (L → Y) had a lowered oxygen affinity, with an off rate 50 times *higher* than that of the wild type, suggesting that tyrosine B10 was unlikely to contribute the high oxygen affinity of *Ascaris* hemoglobin (7).

Ascaris hemoglobin consists of eight identical subunits, each containing two myoglobin-like domains in tandem, followed by a repetitive sequence of polar residues suggestive of a "zipper" (4, 8). Kloek *et al.* (9) made recombinant single domains of *Ascaris* hemoglobin. They found the isolated domains to have an oxygen affinity as high as that of the intact octamer, which proved that the affinity is intrinsic and independent of subunit interaction (9).

We decided to test the possible contribution of tyrosine B10 to oxygen binding in *Ascaris* hemoglobin despite the negative result of the B10 L → Y replacement in myoglobin. Using *Ascaris* hemoglobin cDNA, kindly presented to us by D. E. Goldberg (Washington University School of Medicine, St. Louis), we have synthesized recombinant *Ascaris* globin domains in *Escherichia coli* and replaced tyrosine B10 in the N-terminal domain by either phenylalanine or leucine. We then compared the oxygen- and carbon monoxide-binding properties of the mutant and wild-type domains.

MATERIALS AND METHODS

Our forward PCR primers contained an *Xba* I site, a ribosome-binding site from the T7 phage φ10 gene, an ATG codon, and the first 30 nucleotides of the respective cDNA. The reverse primers contained the last 30 coding nucleotides of each domain, a stop codon, and a *Hin*dIII restriction site. A cDNA sequence encoding a single or double globin domain was amplified by PCR with *Taq* DNA polymerase and was cloned into an M13mp19 vector (10). Site-directed mutagenesis was carried out by the *dut*$^-$, *ung*$^-$ method with *E. coli* BW313 (11). After the DNA sequences of these clones had been confirmed by single-stranded sequencing (12) using Sequenase (United States Biochemical), the *Xba* I–*Hin*dIII fragment was excised and cloned between the *Xba* I and *Hin*dIII sites of a T7 RNA polymerase expression vector, pRK172 (13). The BL21(DE3) strain was transformed with these vectors, and a large-scale culture was carried out at 37°C in 2× TY medium containing ampicillin (100 μg/ml). Cells were harvested after induction with 1 mM isopropyl β-D-thiogalactopyranoside for 3 hr and suspended in 10 mM

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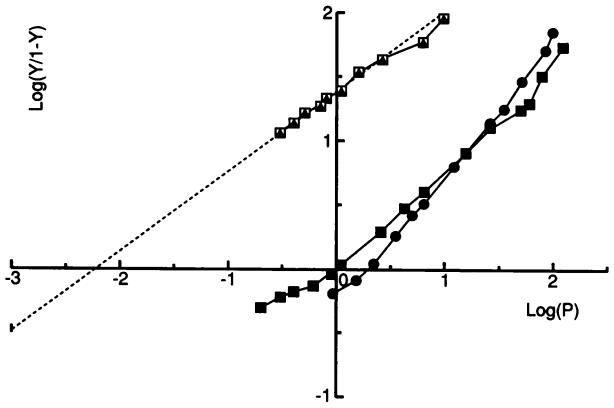

FIG. 1. Hill plots of the oxygen equilibria of *Ascaris* hemoglobin domain 1 wild type (▲) and mutant B10 Y → L (■), and of human α subunits (●). Conditions are stated under Table 1.

sodium phosphate buffer (pH 6.3). The cell lysate was prepared by sonication, adjusted to pH 6.3 with 4 M phosphoric acid, and diluted with cold water until the conductivity was below 2 mS (14). After centrifugation at 35,000 rpm in a Beckman 45Ti rotor, the supernatant was loaded onto a CM-Sepharose CL-6B (Pharmacia) column (4 cm × 10 cm) that had been equilibrated with 10 mM sodium phosphate buffer (pH 6.3), and the protein was eluted with a linear gradient of NaCl from 0 to 100 mM. A second chromatography on CM-Sepharose was essential for purification. Several red peaks were eluted; the peak fractions with the highest A_{412}/A_{280} ratios were pooled and further purified on a Sephacryl S-200 column (4 cm × 100 cm) in 10 mM sodium phosphate buffer (pH 6.3).

All spectrophotometric recordings were performed with an SLM-Aminco DW 2000 apparatus. Fluorescence spectra were obtained with an SLM-Aminco 8000 spectrofluorimeter. The mutant domain 1 with B10 Y → F was difficult to handle, as it tended to precipitate at concentrations over 2 mM and to autoxidize rapidly. For this mutant we first deoxygenated the solution and then equilibrated it under carbon monoxide (1 atm, 101.2 kPa) in the cold. After 5–10 min the static spectrum was recorded and the amount of methemes was calculated. A trace of a freshly prepared 10 mM solution of sodium dithionite was then injected into the optical cuvette and a second spectrum was recorded. The sample was then aerated and used as such (i.e., without passing the sample through a Sephadex G-25 column to avoid denaturation). Oxygen equilibrium curves were obtained with a continuous method (15). In view of the high affinity of these hemoglobin, the zero Po_2 value was checked at the end of the experiments by adding a small amount of buffered sodium dithionite, which also gave the actual absorbance change (ΔA) at zero Po_2. P_{50} and n_{50} were calculated from linear regression analysis according to the Hill equation. The oxygen affinity of the *Ascaris* domain 1 wild-type was so high that at Po_2 values lower than 1 mmHg it was still 90% saturated. Its P_{50} value was found by extrapolation after adjustment of the observed slope of the Hill graph to unity.

Kinetics of carbon monoxide and oxygen binding to the *Ascaris* hemoglobins were obtained after flash photolysis using a 10-ns pulse providing 160 mJ at 532 nm (Quantel yttrium–aluminum garnet laser, France). Samples were 0.1 mM heme in 1-mm/4-mm cuvettes with observation at 436 nm.

To measure the oxygen off rate [$k_{off}(O_2)$], an optical cuvette filled with 100 mM phosphate buffer (pH 7.4) at 25°C was deoxygenated under a stream of argon and a trace of a 2 mM sodium dithionite solution. Then an aliquot of the stock solution of *Ascaris* wild-type domain 1 was injected into the cuvette and the oxygen dissociation optical signal at 415 nm and at 700 nm was recorded as a function of time. The $k_{off}(O_2)$ value was calculated from the curve. This $k_{off}(O_2)$ value and the $k_{on}(O_2)$ measured after flash photolysis allowed us to estimate P_{50} for the domain 1 wild type (at 298 K), which was in good agreement with the value given for native wild type *Ascaris* hemoglobin (2). For the *Ascaris* hemoglobin mutants, the oxygen off rates were measured with a stopped-flow apparatus (Biologic, France) by carbon monoxide replacement; the detection was at 420 nm (6).

Table 1. Oxygen equilibrium parameters for *Ascaris* hemoglobin domain 1 wild type and mutants B10 Y → L and B10 Y → F as compared with tetrameric and monomeric human hemoglobins

Species	P_{50}, mmHg	n_{50}	P_{50} α/P_{50} x
Human α chains*	1.9	0.9	1
Human Hb A (R state)*	21.4	2.76	0.09
Ascaris domain 1			
Wild type*	0.0072	ND	262
B10 Y → L*	0.82	0.8	2.3
B10 Y → F†	0.6 ± 0.5	0.86 ± 0.03	3.1

Experimental conditions were 50 mM [bis(2-hydroxyethyl)imino]-tris(hydroxymethyl)methane (Bistris), pH 6.5/0.1 M NaCl/50 μM EDTA/20 μM catalase/40–60 μM heme, T = 37°C (310 K). ND, not determined.
*Mean of two experiments.
†Mean ± SEM of five experiments from two different preparations.

RESULTS AND DISCUSSION

Fig. 1 shows Hill plots of the oxygen equilibrium curves for the N-terminal *Ascaris* wild-type domain, its mutant B10 Y → L, and human α-globin chains. The curve for the mutant B10 Y → F (not shown) was almost superimposable on that for B10 Y → L. Table 1 lists the values of P_{50} and n_{50}. P_{50} for the wild-type domain agrees with Davenport's (2) for the whole protein if corrected for the temperature difference (his 22°C as against our 37°C). The P_{50} values for the two mutant domains are close to the value of P_{50} for human α chains. The large standard error for the mutant B10 Y → F may be due to its heterogeneity and its instability during our measurements. Table 2 gives $k_{on}(O_2)$, $k_{on}(CO)$, and $k_{off}(O_2)$ compared with those of myoglobin and hemoglobin. These results confirm the slow off rate of domain 1 found by Gibson *et al.* (7). The substitution B10 Y → L accelerates the off rate to a value close to that of sperm whale myoglobin (Table 2). The on rates for oxygen and for CO after flash are also raised but

Table 2. Association rates of carbon monoxide and of oxygen and dissociation rate of oxygen for *Ascaris* hemoglobin domain 1 wild type and mutants B10 Y → L and B10 Y → F

Species	$k_{on}(CO)$, 10^6 $M^{-1}\cdot s^{-1}$	$k_{on}(O_2)$, 10^6 $M^{-1}\cdot s^{-1}$	$k_{off}(O_2)$, s^{-1}
Ascaris domain 1			
Wild type*	0.35	2.8	0.013
B10 Y → L*	0.75	9.0	5
B10 Y → F*	2.7	40.3	2
Sperm whale myoglobin B10 L†	0.55	15	14
Human Hb A (R state)			
B10α and B10β L	6.0‡	40‡	50§

Conditions for on rates: 100 mM phosphate buffer (pH 7.4) at 298 K; CO at 100 μM or O_2 at 270 μM. Conditions for oxygen off rate: 50 mM Bistris, pH 6.5/0.1 M NaCl/50 μM EDTA/20 μM catalase at 298 K. k_{off} values were calculated from the measured k_{on} and P_{50} values.
*Reconstituted domain 1 of *Ascaris* hemoglobin.
†Value from ref. 7, at 295 K.
‡Conditions: 50 mM Bistris, pH 6.5/100 mM NaCl at 298 K (this laboratory).
§From ref. 16, in 50 mM Bistris, pH 7.0/100 mM NaCl at 295 K.

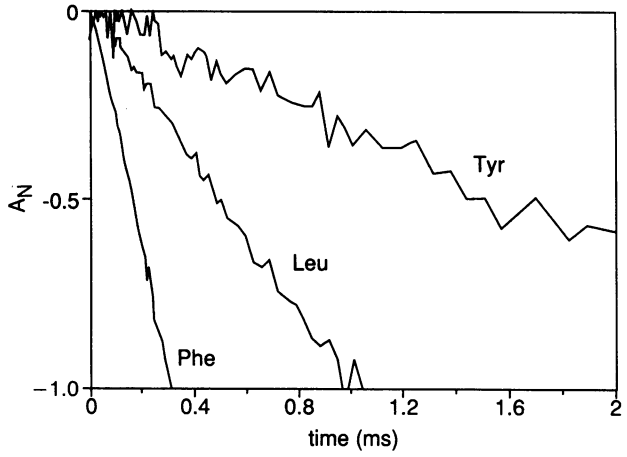

FIG. 2. Recombination kinetics of oxygen after photodissociation of air-saturated solutions of *Ascaris* hemoglobin domain 1 wild type and mutants B10 Y → L and B10 Y → F. A_N on the vertical axis marks the logarithm of the normalized change in absorption at 436 nm.

by much smaller factors than the off rates (Table 2 and Fig. 2).

The mutant hemoglobin B10 Y → L was stable, but the mutant B10 Y → F easily autoxidized, tended to precipitate at higher concentration, and showed strong emission fluorescence at 325 nm, all suggesting an equilibrium between native and unfolded forms, perhaps because the tyrosine hydroxyl is replaced by a water molecule which is a misfit in the heme pocket.

The absorption spectra of *Ascaris* wild-type methemoglobin and of the two mutants Y → L and Y → F at pH 6.4 resemble the spectrum of horse heart metmyoglobin at pH 9 (Fig. 3). This suggests that *Ascaris* methemoglobin at pH 6.4 retains predominantly a hydroxy met (alkaline) species compared with the normal aquo met form present in horse heart myoglobin solutions at acid pH. pH titrations of *Ascaris* methemoglobin domain 1 and of horse heart metmyoglobin revealed pK_a values of 6.5 (this study) and 8.9, respectively. The difference of 2.4 pH units between the pK_a values of the heme-linked hydroxyl in *Ascaris* hemoglobin and horse-heart myoglobin is likely to be due to the hydroxyl in myoglobin being stabilized by a single hydrogen bond from the distal

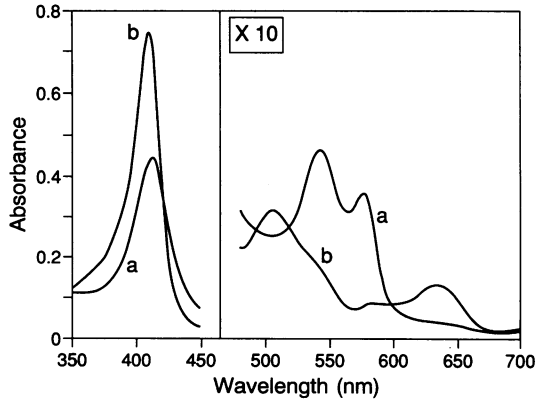

FIG. 3. Static optical spectra of oxidized *Ascaris* domain 1 wild type (spectrum a) and of oxidized horse heart myoglobin (spectrum b) at identical pH of 6.5 in 20 mM phosphate buffer, 4 μM in heme, at 298 K. The spectrum of the *Ascaris* oxidized domain 1 resembles that of a low-spin hydroxymethemoglobin spectrum (low absorbance at 630 nm and high absorbance at 540 nm and 570 nm, together with a large decrease of absorbance of the Soret band, which is shifted toward higher wavelength). By contrast, the spectrum of oxidized myoglobin is typical of high-spin species.

histidine E7, while the hydroxyl in *Ascaris* would be stabilized by two hydrogen bonds, one from the distal glutamine and the other from tyrosine B10. In the B10 Y → L mutant, the gap left by the larger tyrosyl side chain is likely to be filled by a water molecule donating the second hydrogen bond to the heme-linked hydroxyl.

Our results show that a single amino acid replacement, tyrosine in place of the more usual leucine at position B10, is sufficient to account for the high oxygen affinity of *Ascaris* hemoglobin. The 100-fold difference between the P_{50} values of the wild-type and the two mutant *Ascaris* domains corresponds to a difference in binding energy of 2.7 kcal/mol, consistent with the loss of a strong hydrogen bond between the bound oxygen and the hydroxyl of the tyrosine. A second hydrogen bond to the bound oxygen is likely to be donated by the distal glutamine which, in many invertebrates, takes the place of the distal histidine present in most vertebrate hemoglobins (except for elephant myoglobin and the α chain of opossum hemoglobin, which also have distal glutamines, but normal oxygen affinities). The two hydrogen bonds would be sufficient to explain the slow dissociation of oxygen and the consequent high oxygen affinity (Fig. 4).

Why did the recent replacement of leucine B10 by tyrosine in myoglobin by Gibson *et al.* (7) lower the oxygen affinity rather than raise it? The structure of the B10 phenylalanine

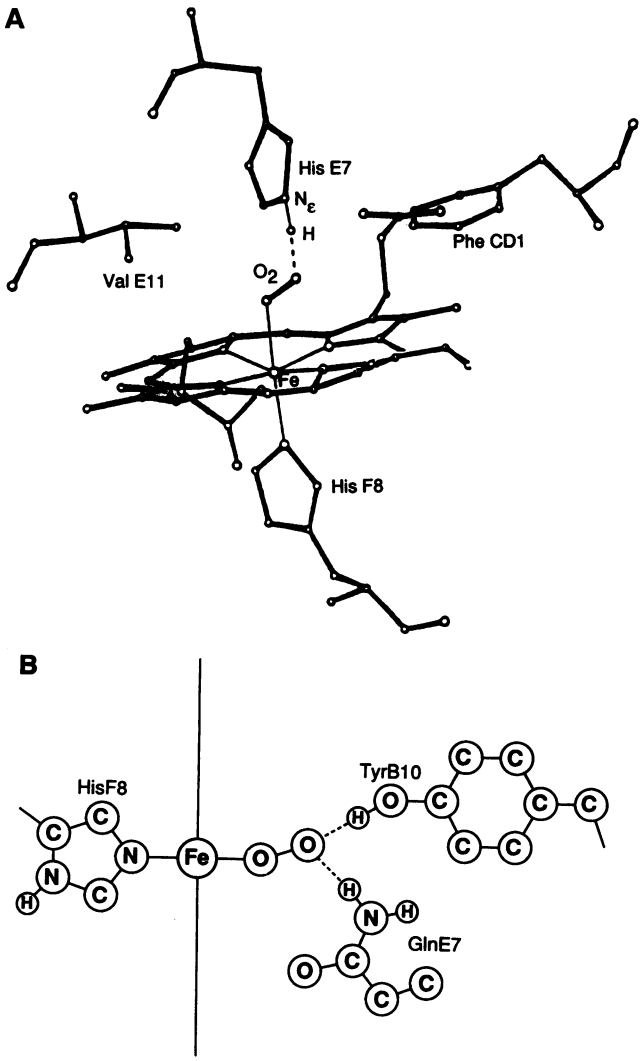

FIG. 4. (*A*) Heme environment in sperm whale oxymyoglobin (24), (*B*) Probable heme environment in *Ascaris* oxyhemoglobin inferred from this study.

mutant determined by Carver *et al.* (6) showed that steric hindrance by the tyrosine hydroxyl would lower the oxygen affinity, because the contact between the benzene ring of phenylalanine B10 and the bound oxygen (CH· · ·O distance, 3.2 Å) is too short to allow a hydroxyl to be interposed without steric hindrance to the iron-bound oxygen. In *Ascaris* hemoglobin, helix B must be 1 Å further away from the heme, so that that hindrance is absent.

There are many other invertebrate hemoglobins with either tyrosine or phenylalanine at position B10. For example, the mollusc *Lucina pectinata*, which lives in symbiosis with H_2S-metabolizing bacteria, has three hemoglobins; two of these have high oxygen affinities with slow off-rates for oxygen, of about 0.1 s^{-1}. Their aquomethemoglobins also have low pK_a values (6.6 and 5.9). Similarity between their EPR spectra and those of the thoroughly studied human hemoglobin M Saskatoon [His E7(63)$\beta \rightarrow$ Tyr] suggested that the slow off rates may be related to the presence of distal tyrosines in their heme pockets, and these have in fact been found in position B10. They also have glutamines in position E7 and phenylalanines in place of the usual valine at E11 (17–20).

Leghemoglobins also have high oxygen affinities, yet in contrast to *Ascaris* and *Lucina* hemoglobins, these are due almost entirely to high on rates. The structures of several derivatives of lupin leghemoglobins show that in oxyhemoglobin the iron-bound oxygen accepts the usual hydrogen bond from the distal histidine and also touches one of the methyl carbons of valine E11 at a C—O distance of 3.2 Å (E. G. Arutyunyan, T. N. Safonova, G. V. Obmolova, A. V. Teplyakov, A. N. Popov, A. A. Rubinsky, I. P. Kuranova, and B. K. Vainshtein, personal communication). All leghemoglobins have either phenylalanine or tyrosine in position B10, but in lupin oxyhemoglobin the phenylalanine is not in contact with the bound oxygen.

Fast on rates require low activation energies in the reaction with oxygen. One of the determinant factors of that energy is the degree of repulsion between the imidazole carbons of the proximal histidine and the porphyrin nitrogens. That repulsion depends on the orientation of the imidazole ring relative to the porphyrin. If the projection of that ring onto the porphyrin plane eclipses the line joining opposite porphyrin nitrogens, repulsion is maximized, but if they are staggered at 45° to each other, repulsion is minimized. The structure of the leghemoglobins suggests a mechanism for maximizing that repulsion in deoxyhemoglobin and minimizing it in oxyhemoglobin.

In deoxyleghemoglobin the imidazole is in equilibrium between two orientations, both close to eclipsed ones where repulsion tends to draw the iron atom out of the porphyrin plane. In oxyhemoglobin, on the other hand, the imidazole rings are in a single orientation close to the staggered one, which facilitates the movement of the iron atom into the porphyrin plane that is optimal for low-spin six-coordinated iron-porphyrin complexes. In this way, coupling of the rotation of imidazole to oxygen binding may lower the activation energy for the transition state.

The examples of the sperm whale myoglobin mutants, *Lucina* hemoglobin, and leghemoglobins show that possession of a tyrosine or phenylalanine at position B10 may be, but need not necessarily be, linked to high oxygen affinity. That link also requires a tertiary structure that brings helix B into juxtaposition to the heme for the correct contact distance between the bound oxygen and the aromatic side chain of residue B10. Could replacement of the distal histidine by glutamine alone raise the oxygen affinity sufficiently? Replacement of the E7 histidine by glutamine in sperm whale myoglobin actually *reduced* the oxygen affinity by a factor of 5–6 (22, 23).

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