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An Exceptional Amino Acid Replacement on the Distal Side of the Iron Atom in Proboscidean Myoglobin

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Summary. Amino acid sequence determination of elephant myoglobin revealed the presence of the unusual substitution E7 His → Gln. Stereochemical analyses suggest that the most suitable residue which can functionally substitute for His at this position in vertebrate globins is Gln. Physiochemical studies imply that the slower rate of autooxidation of elephant myoglobin is the result of this substitution which may confer some selective advantage on the species. Comparative sequence data of paenungulate myoglobins suggest that the His → Gln mutation probably occurred in an ancestor of Elephantinae.

Key words: Myoglobin – Amino acid sequence – Molecular evolution – Elephant

Introduction

Amino acid sequence data can provide insights into the mechanisms of evolution. The sequences are especially informative when x-ray diffraction and kinetic studies have allowed functional significance to be ascribed to specific amino acid sites in a protein. The effect of amino acid substitutions at these functional sites on the adaptive evolution of species using the mutated versions of the protein can then be explored. In myoglobin of modern elephants, we found just such a substitution, the presence of Gln instead of His at position E7. This is the position of the so-called distal histidine.

Of the vertebrate globin sequences studied to date, approximately 230, only five have Gln at E7, namely Asian elephant myoglobin (Dene et al. 1980), African elephant myoglobin (reported here), opossum α-hemoglobin chain (Stenzel et al. 1979) hagfish monomeric globin (Liljeqvist et al. 1979) and the shark, *Mustelus antarcticus* (Fisher et al. 1980). All remaining sequences have His at E7. This residue is intimately associated with the globin molecule's uptake of O_2. During this process its imidazole group is thought to swing away from the heme pocket's entrance, thus permitting access of O_2 to the iron atom (Nobbs 1966; Perutz and Mathews 1966; Takano 1977a,b; Case and Karplus 1979).

In attempting to understand the significance of the E7 His → Gln substitution in elephant myoglobin, we investigated when the mutation occurred in the descent of the Paenungulata, the superorder to which the elephant's order Proboscidea belongs (Simpson 1945). Also, we explored the stereochemical implications in the myoglobin molecule of the seven amino acid substitutions resulting from single point mutations in His codons. Finally, we investigated the mechanism by which E7 Gln in elephant myoglobin affects such functional parameters as oxygen dissociation equilibrium, kinetics of O_2 dissociation and CO association and dissociation, and rate of oxidation. From our findings we suggest that this substitution served an adaptive role in elephant evolution.

Evolutionary History

The primary structure of Asian elephant (*Elephas maximus*) myoglobin is shown in Fig. 1 (Dene et al. 1980). The most important findings about this sequence concerned its marked divergence from other mammalian myoglobins and the presence of Gln rather than His at position E7. Among over 60 known mammalian myoglobin sequences, the elephant's most resembled those of horse and sportive lemur, but still

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 1  2  3  4  5  6  7  8  9  10 11 12 13 14 15 16 17 18 19  20
Gly Leu Ser Asp Gly Glu Trp Glu Leu Val Leu Lys Thr Trp Gly Lys Val Glu Ala Asp

 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39  40
Ile Pro Gly His Gly Glu Phe Val Leu Val Arg Leu Phe Thr Gly His Pro Glu Thr Leu

 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59  60
Glu Lys Phe Asp Lys Phe Lys His Leu Lys Thr Glu Gly Glu Met Lys Ala Ser Glu Asp

                E7
 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79  80
Leu Lys Lys Gln Gly Val Thr Val Leu Thr Ala Leu Gly Gly Ile Leu Lys Lys Lys Gly

 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100
His His Glu Ala Glu Ile Gln Pro Leu Ala Gln Ser His Ala Thr Lys His Lys Ile Pro

101 102 103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120
Ile Lys Tyr Leu Glu Phe Ile Ser Asp Ala Ile Ile His Val Leu Gln Ser Lys His Pro

121 122 123 124 125 126 127 128 129 130 131 132 133 134 135 136 137 138 139 140
Ala Glu Phe Gly Ala Asp Ala Gln Gly Ala Met Lys Lys Ala Leu Glu Leu Phe Arg Asn

141 142 143 144 145 146 147 148 149 150 151 152 153
Asp Ile Ala Ala Lys Tyr Lys Glu Leu Gly Phe Gln Gly
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Fig. 1. Amino acid sequence of the Asian elephant skeletal muscle myoglobin. Asian and African elephant myoglobin both have Gln at position 64 (E7) and only vary from each other at position 129 where African elephant myoglobin has Ala rather than Gly

diverged from each by 22 amino acid differences (Dene et al. 1980). This indicated that the clade containing Proboscidea arose as one of the earliest offshoots of the common eutherian stem, an hypothesis also supported by α-lens crystallin sequences (de Jong et al. 1977). Alternatively, the fixation of a large number of mutations during proboscidean descent could have resulted from a very fast rate of myoglobin evolution, similar to that encountered along the cetacean lineage (Dene et al. 1980; Romero-Herrera et al. 1978). The finding of Gln at position E7 in Asian elephant myoglobin raised the question of whether this mutation was an isolated one in the specimen we studied or whether it was a species characteristic. Rather than screening *Elephas maximus* populations we decided to examine its closest relative, *Loxodonta africana* (the African elephant). Alignment of the tryptic and peptic myoglobin peptides from the latter species against the former revealed only one amino acid difference, a substitution of Ala by Gly at position 129 (see Fig. 1) which was confirmed by sequential studies. Likewise, the presence of Gln at E7 in the African elephant's myoglobin was verified by dansylation.

Elephas maximus and *Loxodonta africana* are the only surviving members of the order Proboscidea. Fossil evidence indicates that Elephantinae, the subfamily that these two species belong to, had its origin about 5 to 6 million years ago in sub-Saharan Africa (Maglio 1974). During the Pliocene and Pleistocene the Elephantinae radiated into new habitats in Africa, Asia, Europe, and North American and gave rise to lineages classified into over 20 species of three genera, *Loxodonta, Elephas,* and *Mammuthus* (Maglio 1972). Although the genus *Mammuthus* is not represented by any living species, *Mammuthus primigenius* (the woolly mammoth) did survive until around 10,000 years ago, inhabiting the cold regions of Siberia and Alaska where not only its skeletal and dental remains, but its soft tissues as well continue, even now, to endure in the permafrost. From work in progress on the frozen mammoth discovered several years ago near Magadan, Siberia (Shilo 1978), it is evident that protein data can be gathered on *Mammuthus primigenius* (Romero-Herrera et al. 1981; Shoshani et al. 1981; Goodman et al. 1980; Prager et al. 1980). Thus it might prove possible to investigate whether all three genera of the Elephantinae share the E7 His → Gln substitution.

The elephants's order Proboscidea is part of a larger clade within the mammalian subclass Eutheria. Immunological data, particularly those obtained with chicken antisera to various mammalian albumins (Shoshani et al. 1981), depict Sirenia (manatee and dugong) as the order genealogically closest to Proboscidea. These two groups are joined by Hyracoidea first (the three extant members of Simpson's superorder Paenungulata) and then by Tubulidentata (aardvark). Evidence from the amino acid sequences of α-lens crystallin chains also point to a monophyletic "paenungulate" origin of aardvark, hyrax, manatee, and elephant (de Jong et al. . In press). Thus, in an attempt to establish the antiquity of the His → Gln substitution at E7, muscles were obtained from manatee (*Trichechus manatus*), hyrax (*Procavia capensis*) and aardvark (*Orycteropus afer*). Their myoglobins were purified, digested with trypsin, and fingerprinted. The resulting patterns, together with those of the African and Asian elephants are shown in Fig. 2.

The tryptic peptide containing residues 64-77, isolated from each of the three non-elephant myoglobins, was subjected to amino acid analysis. This peptide has helical position E7 at its N-terminus. Since His was confirmed at E7 in all three species by dansylation, the E7 His → Gln substitution probably occurred after the ori-

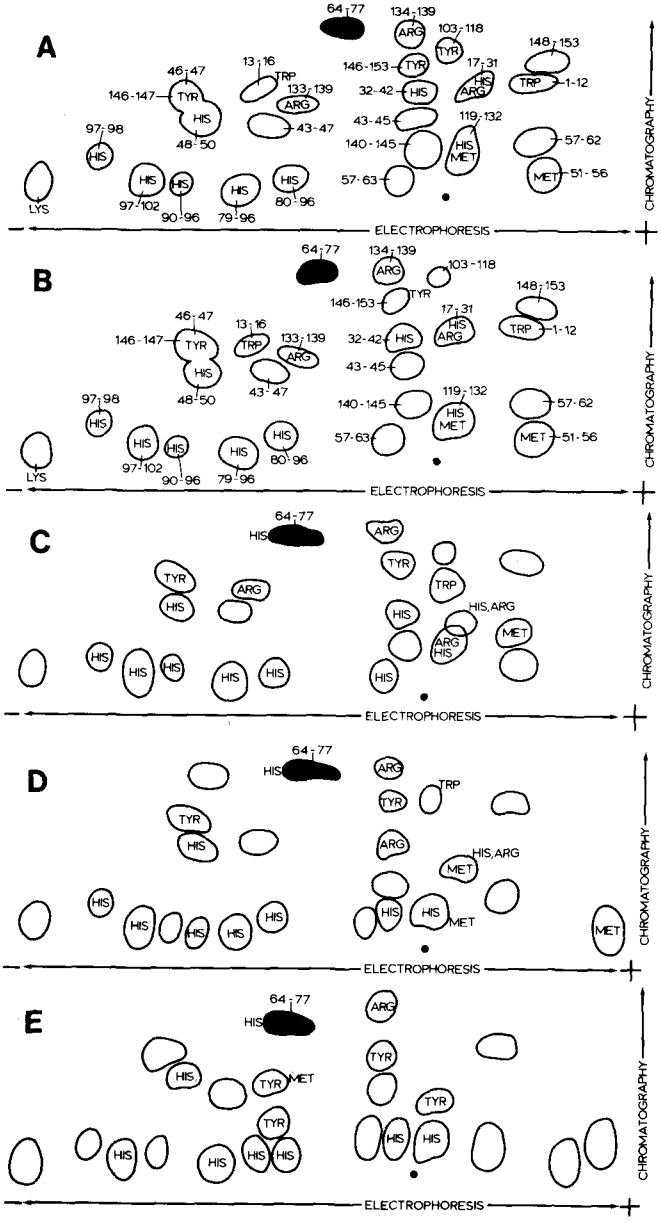

Fig. 2A-E. Fingerprint patterns of the tryptic peptides from myoglobins of Asian elephants (A), African elephant (B), hyrax (C), aardvark (D), and manatee (E). Staining reactions for Trp, Tyr, Arg, His, and Met are indicated. The point of application for the sample is indicated by a dot. The peptide containing the E7 position has been darkened. These fingerprints have been placed in descending order (A-E) according to decreasing degree of similarity. Except for the identity between the two elephants, these fingerprints do not reflect the present consensus of opinion on phylogenetic relationships. Although manatee (E) is generally considered to be the closest living relative to elephants (A and B), its fingerprint pattern appears the most divergent. This may be a reflection of a very fast rate of myoglobin evolution like that found for cetaceans

gin of Proboscidea either in an ancestor or early member of Elephantinae.

Stereochemical Analysis

Not only has His been overwhelmingly favored at position E7 of vertebrate globins but the only alternative residue fixed during evolution seems to be Gln. X-ray diffraction studies have shown that the side chain of His can close the gap which separates the α-carbon of E7 from the iron atom of the heme prosthetic group. Although the mechanisms of interaction between His and various ligands are known (Perutz and Mathews 1966; Stryer et al. 1964; Hendrickson and Love 1971; Yonetani et al. 1974; Norvell et al. 1975; Ikeda-Saito et al. 1977), the precise interplay between this residue and oxygen has not yet been fully established (Ikeda-Saito et al. 1977; Tucker et al. 1978). Studies based on molecular dynamics calculations (Case and Karplus 1979) suggest that the stereochemically and energetically most favored route for oxygen entrance into the heme pocket is between residues E11 Val or E10 Thr and E7 His. This pathway is thought to result from a conformational change along the E helix backbone Takano 1977a, b; Frauenfelder et al. 1979). Theoretical considerations predict that a displacement of the distal His imidazole ring, as the result of a rotational movement of the C^{α} - C^{β} and/or C^{β} - C^{γ} bonds, allows for the final access of oxygen to the iron atom (Nobbs 1966; Chance et al. 1966; Case and Karplus 1979).

Using the X-ray diffraction coordinates of sperm whale deoxymyoglobin (Takano 1977b) and tridimensional displays on a MMS-X Graphic System, we examined the distances between the heme iron atom and all residues at position E7 which could arise by single nucleotide substitutions from His codons (Gln, Asp, Asn, Leu, Tyr, Arg, and Pro). Figure 3 shows the graphic display and atomic distances of the relevant atoms between the mutant variants and the iron atom. His closes the entrance to the heme pocket better than any other residue. The best alternative residue appears to be Gln whose Nϵ1 and Oϵ2 are less than 1 Å from the positions occupied by His Cϵ1 and Nϵ2. Asp, Asn and Leu do not close the gap as well as Gln because their side chains are shorter. Furthermore, the negative charge carried by the Asp Oδ2 may produce an electrostatic repulsion of molecular oxygen. None of these three residues exist at position E7 in the vertebrate globins so far studied. When E7 His is replaced by Tyr as in the human hemoglobin variants Boston and Saskatoon (Gerald and Efron 1958), the hydroxyl group of this residue comes so close to the iron atom that an ionic bond is formed which results in the conversion of the heme iron into the ferric state (Perutz and Lehmann 1968). In the graphic display shown in Fig. 3, the large side chain of Arg can be made to fit within the myoglobin heme pocket by assuming a

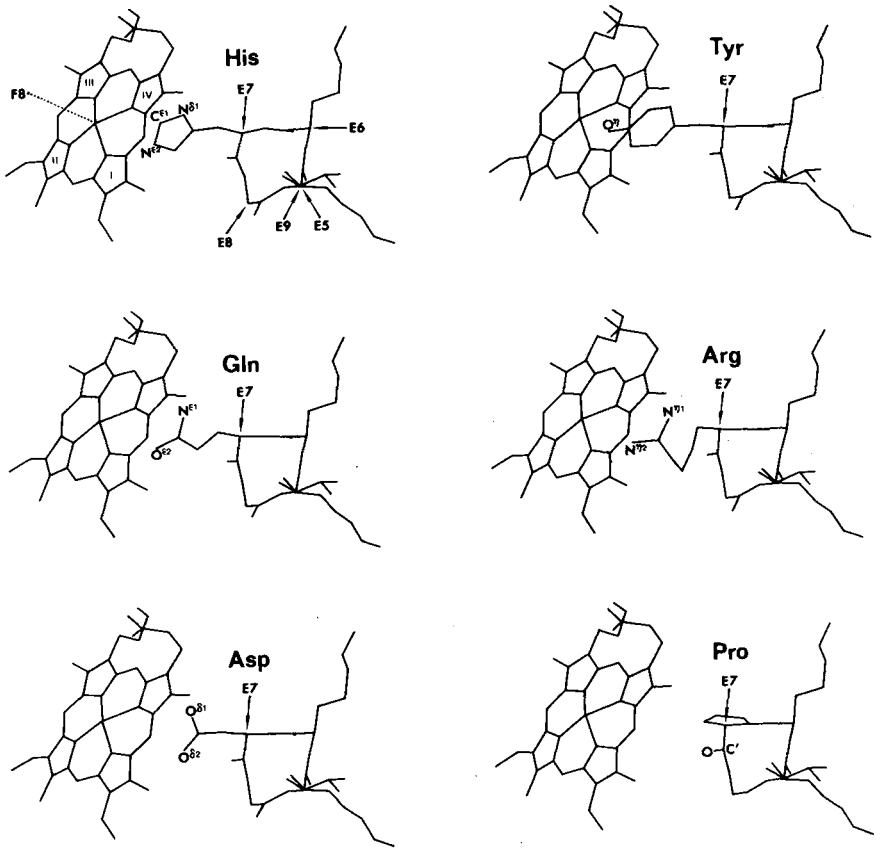

Fig. 3. Stereochemical patterns of the heme prosthetic group and residues E5 to E9 obtained from the crystallographic coordinates for sperm whale deoxymyoglobin (Takano 1977a, b). The X-ray diffraction coordinates were provided by the Protein Data Bank (Chemistry Department, Brookhaven National Laboratory), and displayed on a MMS-X Graphic System. These views show His and five other amino acid residues at E7 that can arise from His codons by single point mutations. Since Asn and Leu have stereochemical patterns similar to Asp, their views are not shown. In order to obtain the same spatial orientation on the screen, the α-carbons of the residues at E5 and E9 were made to coincide

spring-like configuration. A mutation placing Arg at position E7 is present in hemoglobin Zürich (Muller and Kingma 1961). Recently, Tucker and colleagues (1978) demonstrated that the long aliphatic side chain of Arg protrudes toward the surface of the molecule probably forming a salt bridge with one of the heme propionic groups. Although this variant still combines with oxygen, the large gap left in front of the heme makes the iron atom easily oxidizable. If ever Arg is found at position E7 in myoglobin, it willl most likey occupy the same position as in hemoglobin Zürich. Finally, a mutation from His to Pro will leave a large empty space (see Fig. 3) since the closest atom to the heme iron is the E7 carbonyl oxygen of the main chain. This mutation is present in hemoglobin Bicêtre (Wajcman et al. 1976), a very unstable variant producing grave clinical consequences.

Besides being present in elephant myoglobin, E7 Gln also occurs in the monomeric hemoglobin of the hagfish (Liljeqvist et al. 1979) and in the α-hemoglobin chain of the Virginia oppossum (Stenzel et al. 1979) and it has recently come to our attention that the shark, *Mustelus antarcticus* also has an E7 Gln (Fisher et al. 1980). Hagfish globin in contrast to elephant myoglobin, shows numerous differences from other known globin chains along the E-helix and at the crossing of the B and E helices. Whereas close contact is normally established between residues B6 Gly and E8 Gly in most globins, the hagfish globin has Gln and Ala respectively. Such bulkier residues are likely to alter some of the stereochemical parameters of the E-helix. A similar situation occurs with the opossum hemoglobin α-chain in which position B6 is Met rather than Gly. It has been suggested that such a substitution, together with that at position B13 (Thr in the opossum, Met in the rest of the known α-chain hemoglobins) was compensated for by the presence of Gln at position E7 in opossum (Stenzel et al. 1979).

In the case of elephant myoglobin none of these conclusions are applicable because, with the exception of E7, this protein has the same E-helix sequence found in sperm whale myoglobin. Furthermore, there are only three non-conservative differences between sperm whale and elephant myoglobins (Dene et al. 1980) and these are distant from the active center and at the surface of the molecule (positions A11, B8 and B16).

It is worth noting that in evolutionary terms E7 Gln will either persist or mutate back to His. It can also change by single point mutations to Leu, Pro, Arg, Lys, or Glu. However, none of these alternative residues are as suitable as Gln at E7. The first three substitutions have already been discussed. Lys like Arg would probably be oriented towards the surface of the molecule leaving a large gap in front of the heme iron. Glu placed in the same steric position as His would form a salt bridge between its side chain carboxyl oxygen and the iron atom.

Kinetic and Equilibrium Studies

The physiological significance and thus possible adaptive role of E7 Gln in elephant myoglobin was examined with regard to ligand interactions and rate of heme oxidation. For purposes of comparison human myoglobin, which has E7 His (Romero-Herrera and Lehmann 1974), was examined in parallel with elephant myoglobin. Purification of both myoglobins was carried out by standard procedures (Romero-Herrera and Lehmann 1974) at 4°C (without the inclusion of KCN) except that column chromatography was performed on DE-52 cellulose (50 mM TRIS-HCl pH 8.42).

The oxygen dissociation equilibrium isotherms of both elephant and human myoglobins are hyperbolic and nearly identical (see Fig. 4A). Both myoglobins have a Hill constant of 1.19. The P_{50} value for human myoglobin is 0.60 mm Hg and for elephant myoglobin 0.62 mm Hg. The former results are in agreement with those of other investigators (Antonini and Brunori 1971). Furthermore, the oxygen dissociation rate constant from both species are also similar, 19.4 sec^{-1} and 18.4 sec^{-1}

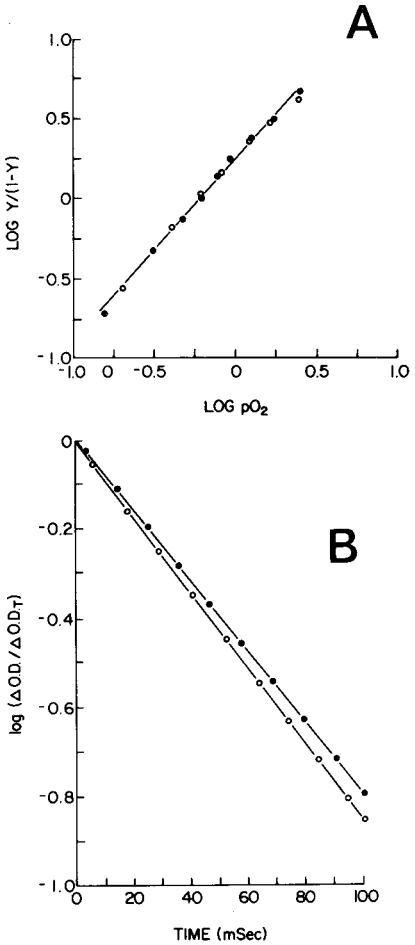

Fig. 4. A Hill plots of the oxygen dissociation equilibrium of elephant (0) and human (•) myoglobins. The purified and partially oxidized myoglobins were reduced with sodium dithionite under anaerobic conditions and the reductant removed by gel filtration (Sephadex G25, 0.1M phosphate buffer, pH 7.4). The equilibrium curves were obtained by tonometer methods (Eliot and Mizukami 1977) at 22°C. Myoglobin concentration was 0.15 mM.

B First order kinetic plots for O_2 dissociation of elephant (0) and human (•) myoglobins. These measurements were determined using an Aminco-Morrow stopped flow apparatus at 22°C in the presence of sodium dithionite. The myoglobin concentration was 0.1 mM in 0.1M phosphate buffer, pH 7.4. The spectral change was recorded at 581 nm. The dissociation rates between the myoglobins did not significantly vary between pH 6.5 and 8.5

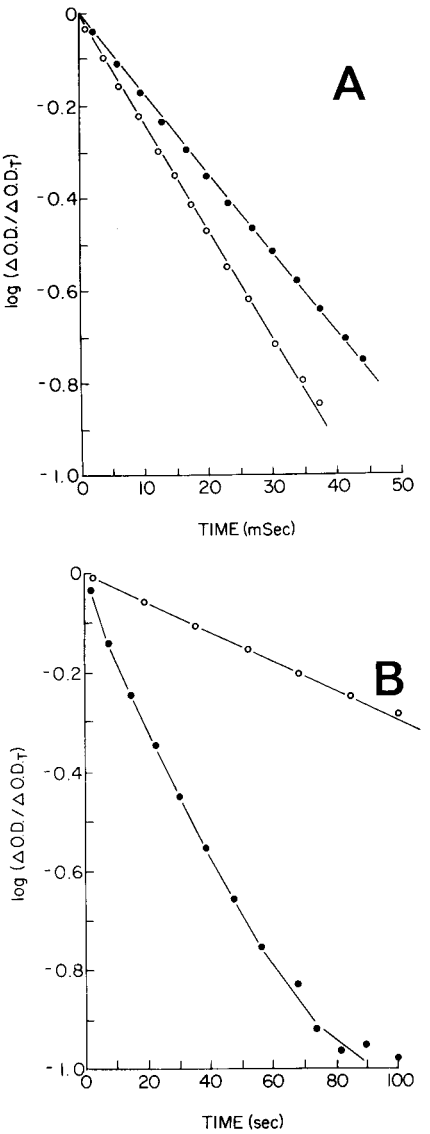

Fig. 5. A CO-association, B CO-dissociation rates of elephant (0) and human (•) myoglobins. These determinations were made using the same stopped-flow apparatus as Fig. 4B at 22°C in 0.1 M phosphate buffer, pH 7.4. CO and myoglobin concentrations were 0.1 mM and 0.02 mM, respectively. The apparent association rate constant was 52.9 sec^{-1} for elephant myoglobin and 40.0 sec^{-1} for human myoglobin. The CO-dissociation rate was determined by NO replacement utilizing a myoglobin concentration of 0.011 mM. The K_{off} = 0.0068 sec^{-1} for elephant myoglobin and 0.032 sec^{-1} for human myoglobin

for human and elephant myoglobins, respectively (Fig. 4B). On the other hand, elephant myoglobin has a CO association rate approximately 25% greater than that for human myoglobin and a dissociation rate that is almost five times slower than that of human myoglobin (Fig. 5A and B). Electrostatic interaction between the amide oxygen of Gln E7 and the carbon of a polar CO ligand, as well as the possibility that a strong hydrogen bond may form between the amide hydrogen and the oxygen of the ligand could be responsible for the enhanced CO-binding in elephant myoglobin. These interactions may be less important for O_2. The steric differences between Gln and His may be of greater consequence for CO binding than for O_2 binding, since CO preferentially assumes a perpendicular axis with the heme plane, while O_2 more easily forms a bent configuration away from the distal residue (Moffat et al. 1979).

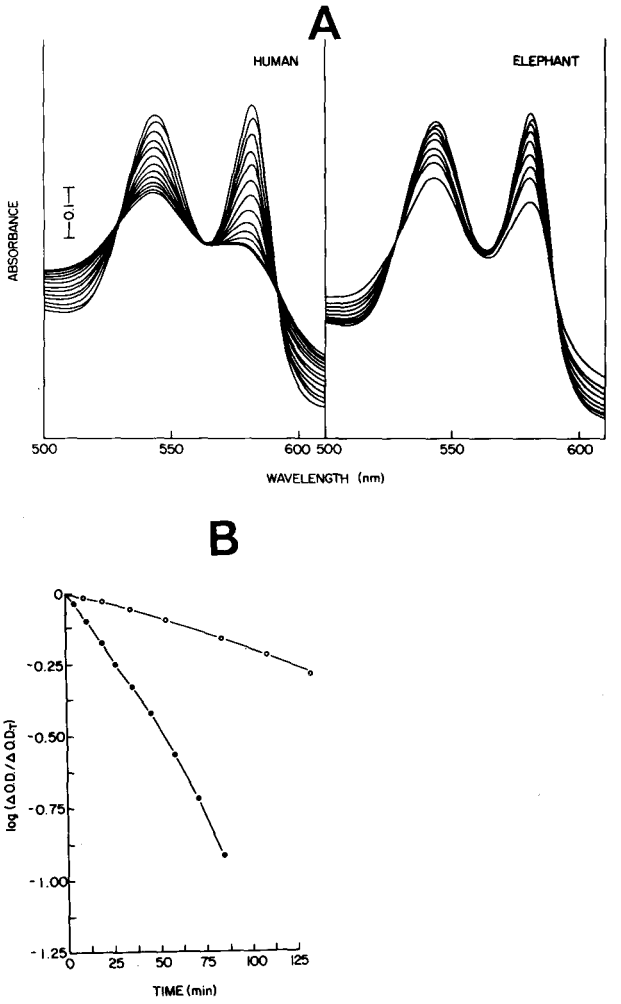

Fig. 6. A Consecutive optical spectra of elephant and human oxymyoglobins undergoing autooxidation in the presence of azide at 22°C in 0.1M phosphate buffer, pH 7.0. The myoglobin concentraton was 0.22 mM.

B The normalized time course of the reaction shown in A. The apparent rate constant for elephant myoglobin was 7.86×10^{-5} sec^{-1} and for human myoglobin was 3.55×10^{-4} sec^{-1}

Usually, in hemoglobin, replacements at E7 are accompanied by accelerated heme oxidation in vitro (Antonini and Brunori 1971; Wallace et al. 1974). We observed, however, that the elephant myoglobin was very resistant to oxidation during the purification process in spite of the fact that no protecting ligand was used. The slow rate of conversion into the ferric state was spectrophotometrically measured by using sodium azide as the displacing ligand (Fig. 6A and B). Elephant myoglobin reacted four-and-a-half times slower than human myoglobin in forming azide metmyoglobin. Preliminary studies using other oxidizing agents (e.g., hydroquinone, NO_2^-) have also confirmed these results. Autoxidation of oxymyoglobin by azide is thought to involve protonation of the distal oxygen of the O_2 ligand followed by reductive displacement of the superoxide (Wallace et al. 1974). The distal Gln either through hydrogen bonding of its amide hydrogen to the distal ligand oxygen or through electrostatic repulsion of its amide oxygen from the ligand oxygen might well hinder superoxide formation. Obviously, without the presence of a strong nucleophilic anion, autoxidation would proceed more slowly. Since elephant myoglobin lacks a readily protonated group, the rate of autoxidation would be even slower than in myoglobins having His at E7.

It should be noted that elephant myoglobin has an E helix which differs from the human only at positions E7 and E9 and from the sperm whale only at positions E7 and E17. Inasmuch as E9 and E17 are both external and E9 additionally is a highly variable site (Romero-Herrera et al. 1978), it is unlikely that these positions have significant roles with respect to the heme environment. Therefore, because some of our more recent studies indicate that sperm whale myoglobin differs functionally from the elephant molecule in the same manner as does human myoglobin (Bartnicki et al. 1980), it can be concluded that the His → Gln substitution at E7 is solely responsible for the different kinetic properties of elephant myoglobin with regard to CO association-dissociation and rates of autoxidation.

Possible Evolutionary Significance

Elephants and whales, the largest terrestrial and marine animals respectively, both have myoglobins which accumulated far more than the average number of amino acid substitutions (Dene et al. 1980; Romero-Herrera et al. 1978). It is probable that the high number of fixed mutations along the cetacean lineage enhanced the ability of myoglobin to readily supply oxygen to muscle mitochondria during long periods of diving (Romero-Herrera et al. 1978). The storage and supply of oxygen is further facilitated by the unusually large concentration of myoglobin in cetacean skeletal muscles (Wittenberg 1970). Elephants also require large amounts

of energy for their skeletal muscles, in this case to maintain posture against gravity. Nevertheless the concentration of their myoglobin falls within the normal range for other mammals (Wittenberg 1970). This suggests that elephant myoglobin may have some special adaptive advantage which resides among its amino acid substitutions. Presumably the mutation at E7 is the crucial one because none of the substitutions which occurred at other positions in this myoglobin would be likely to affect the heme environment (Dene et al. 1980). Moreover the replacement of His by Gln occurred despite the general tendency during vertebrate evolution for His to be favored at position E7 in the globin family rather than any other amino acid residue.

We have demonstrated that elephant myoglobin, while resembling typical mammalian myoglobins in oxygen carrying parameters, does have a several fold greater resistance to oxidation. Even though, in vitro, metmyoglobin reductase is extremely efficient ($K_m = 5 \times 10^{-5}M$) in restoring the ferrous state (Hagler 1979), in vivo this may not always be so considering the viscosity and architecture of the muscle cell as well as the random distances separating enzyme from substrate. Therefore, a mutation such as His → Gln at E7 which apparently prevents heme oxidation might well offer some positive selective advantage especially in a system with high oxygen demands. Alternatively, we might speculate that the His → Gln substitution in elephant myoglobin compensated for a slightly deleterious mutation which affected either the activity or the quantity of the elephant's metmyoglobin reductase. Such a mutant metmyoglobin reductase gene, despite its negative coefficient of fitness, could still be fixed if it were linked to some other mutant gene or genes being strongly selected.

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