Volume 282, number 2, 281–284 FEBS 09670 May 1991

ADONIS 001457939100377Z

Control and recognition of anionic ligands in myoglobin

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Received 1 March 1991

Equilibrium and kinetic experiments on site-directed mutants of a synthetic sperm whale myoglobin (Mb) gene have been performed. Results on the reactivity on both ferric and ferrous wild type and mutants Mb's are presented. Analysis of ligand binding to *His(E7)Val* and *His(E7)Val-Thr(E10)Arg* mutants compared to wild-type sperm whale, horse and *Aplysia limacina* Mb's, shows that the introduction of an arginyl residue at the topological position E10 greatly enhances the stability of the various Mb:heme ligand adducts. Alternative mechanisms of ligand stabilization may therefore be operative in Mb's lacking the distal histidine.

Protein engineering; Myoglobin mutant; Ligand binding

1. INTRODUCTION

Control of ligand binding in myoglobin and hemoglobin is exerted via heme pocket proximal and distal amino acid residues [1–3]. On the distal side a major determinant of reactivity is the imidazolyl residue of His^{64} (E7), which controls the rate of O_2 dissociation by hydrogen bonding. This interaction was shown by neutron diffraction data on sperm whale MbO_2 [4], and supported by kinetic experiments on artificial mutants [5]. Using a synthetic sperm whale Mb gene [6], His(E7) has been replaced by several amino acid residues: while Gln can cope with the task of stabilizing the bound O_2, for other residues tested (e.g. Gly and Val) the O_2 dissociation rate constant is increased by 10^2–10^4 times [7]. For globins lacking the distal histidine, alternative mechanisms of stabilization of the ligand bound at the 6th coordination position of the heme iron may be operative. The inspection of the three-dimensional structure of the F^- derivative of *Aplysia limacina* Mb (hereafter *A. limacina* Mb) [8], containing a valyl residue at position E7, indicates that Arg(E10), which is exposed to the solvent in ferric Mb [9], swings into the pocket interacting with the heme bound F^-. The corresponding residue in sperm whale Mb, Thr(E10), cannot exert the same role. Thus, the stabilization of *A. limacina* MbO_2, as documented by the relatively slow oxygen dissociation rate constant ($k = 70\ s^{-1}$) [10] was likewise attributed to Arg(E10) [8]. These considerations prompted us to test this structural hypothesis by equilibrium and kinetic experiments on site-directed mutants of sperm whale Mb. The results reported below indicate that the sperm whale Mb mutant *His(E7)Val* has a very high ligand dissociation rate constant not only for O_2 but also for N_3^-; next, the double mutant *His(E7)Val-Thr(E10)Arg* displays considerably slower dissociation rate constants. These data support the role of Arg(E10) in stabilizing the heme bound ligands. Additional engineering of the distal pocket will be necessary in order to attain full stability of the liganded state.

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Abbreviations: Mb, myoglobin

2. MATERIALS AND METHODS

Mutagenesis of the synthetic sperm whale Mb gene [6] was performed using cassette mutagenesis with a set of oligonucleotides where the normal codons for His (CAT) and Thr (ACC) have been substituted with Val (GTT) and/or Arg (AGG), respectively. DNA manipulations were essentially as described by Maniatis et al. [11]. DNA sequencing has been carried out as described by Hattory and Sasaki [12] using Bethesda Research Laboratories reagents. Wild-type and mutant sperm whale Mb's were expressed in *E. coli* and purified to homogeneity as previously described [6] with the following minor modification: the ion-exchange column was a CM-Sepharose Fast Flow (Pharmacia) eluted with a linear gradient of 50–250 mM NaCl in 15 mM phosphate buffer, pH 6.0. Horse Mb was obtained from Sigma and further purified by ammonium sulphate precipitation [10].

Equilibrium constants for N_3^-, F^- and OH^- binding were obtained spectrophotometrically [10], using a Varian Cary 219 double beam spectrophotometer. Kinetics for N_3^- binding were measured by (i) the temperature jump relaxation method [13,14], using an instrument built by Messanlagen Studiengesellschaft mbH (Göttingen, Ger-

Table I

Equilibrium and/or kinetic parameters for the binding of N_3^-, F^- and OH^- to wild-type and mutant ferric myoglobins

Myoglobin	N_3^-			F^-	OH^-
	k' ($M^{-1}\cdot s^{-1}$)	k (s^{-1})	K (M^{-1})	K (M^{-1})	pK_a
Horse	2.6×10^3 [b]	3.8×10^{-1} [b]	4.5×10^4 [c]	6.3×10^3 [c,d]	8.8
Sperm whale[e]	2.5×10^3 [b]	5.0×10^{-1} [b]	3.7×10^4 [c]	6.2×10^3 [c]	9.0
His(E7)Val	2.6×10^3 [f]	7.4×10^2 [f]	3.2×10^2 [c]	<1 [c]	10.2
His(E7)Val-Thr(E10)Arg	3.0×10^3 [f]	4.8×10^1 [f]	1.6×10^4 [c]	2.0×10^3 [c]	8.8

[a] According to literature [10], values of the equilibrium constant (M) for OH^- binding are quoted as pK_a. Values of pK_a were determined at 20°C, in 0.2 M acetate buffer (from pH 5.0 to 6.0), 0.1 M phosphate buffer (from pH 6.0 to 8.0), 0.2 M Tris (hydroxymethyl)aminomethane-HCl buffer (from pH 7.5 to 9.0), 0.2 M borate buffer (from pH 8.0 and 10.0), and 0.2 M carbonate buffer (from pH 9.0 to 11.0).
[b] Values of k' and k were determined by rapid-mixing stopped-flow experiments at 20°C, in 0.1 M phosphate buffer, pH 7.0.
[c] Values of K were obtained at equilibrium at 20°C, in 0.1 M phosphate buffer, pH 7.0.
[d] From ref. 19.
[e] Synthetic wild-type sperm whale Mb.
[f] Values of k' and k were obtained by temperature jump experiments at 25°C, in 0.1 M phosphate buffer, pH 7.0.

many); and (ii) the stopped-flow method [10], carried out with a Durrum-Gibson apparatus. Kinetics for O_2 and CO binding were estimated by flash photolysis experiments [10], with an apparatus similar to that described by Brunori and Giacometti [15]. Under all experimental conditions, the observed kinetic processes conformed to a single exponential event.

3. RESULTS AND DISCUSSION

Values of the equilibrium and/or kinetic parameters for N_3^-, F^- and OH^- binding to the wild-type and to the sperm whale Mb mutants *His(E7)Val* and *His(E7)Val-Thr(E10)Arg* are shown in Table I by comparison with those for wild-type sperm whale and horse Mb's, which are generally considered as molecular models for monomeric hemoproteins [10]. The *His(E7)Val* mutant displays a low affinity for N_3^-, F^- and OH^-; when the second mutation (Thr(E10)Arg) is introduced, a clear-cut increase in affinity (about 20-fold) is observed.

The role of Arg(E10) is best illustrated by comparing the rate constants for the formation and the dissociation of the N_3^- derivative of different hemoproteins (see Table I). Under all the experimental conditions, kinetics of N_3^- binding conforms to a simple process quantitatively consistent with the following reaction scheme:

$$Mb^+ + N_3^- \underset{k}{\overset{k'}{\rightleftharpoons}} Mb^+ N_3^-$$

with the relaxation time τ being described by the following relation (Eqn. 1 [13,16]):

$$\tau^{-1} = k + k' ([Mb^+] + [N_3^-]) \quad (1)$$

This finding is proven by the results reported in Fig. 1, which conform to Eqn. 1 within experimental errors.

Data shown in Table I allow the following considerations.

(i) The bimolecular rate constant for the sperm whale $Mb^+N_3^-$ complex formation (k') is about 100-fold faster in the two mutants as compared to the wild type. Indeed in the latter case the presence of His(E7) stabilizes a water molecule at the 6th coordination position of the heme iron, and therefore N_3^- binding re-

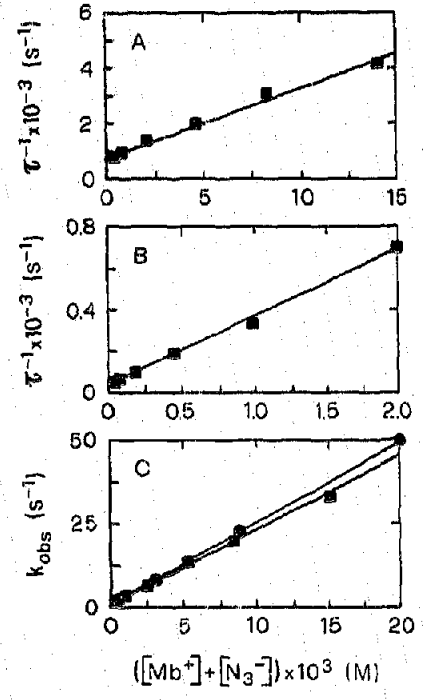

Fig. 1 Dependence of τ^{-1} on the equilibrium concentration of reagents ([Mb^+] + [N_3^-]; see Eqn. 1), as obtained by temperature jump experiments at 25°C, for N_3^- binding to the *His(E7)Val* and *His(E7)Val-Thr(E10)Arg* mutants of sperm whale Mb (panel A and panel B, respectively). Dependence of the apparent rate constant (k_{obs}) on the concentration of reagents ([Mb^+] + [N_3^-]), as obtained by stopped-flow experiments at 20°C, for N_3^- binding to horse and wild-type sperm whale Mb's (panel C; circles and squares, respectively). Straight lines are linear regressions of data points. Data were obtained at Mb concentration = 4.0×10^{-6} M in 0.1 M phosphate buffer, pH 7.0.

quires ligand substitution, which slows down the process [14,17]. In the two mutants with Val(E7) no water molecule is coordinated, as inferred also from the optical absorption in the Soret region, where the maximum of the ferric derivative is at 392 nm, with an extinction coefficient of 100 $mM^{-1} \cdot cm^{-1}$; these parameters are very different from those of the wild-type sperm whale Mb [17]. These observations are fully consistent with previous conclusions on *A. limacina* Mb reached on the basis of NMR, kinetic and crystallographic data [9,14,17].

(ii) The rate constant for N_3^- dissociation (k) follows a clear trend, increasing from 0.3 s^{-1} for the wild-type sperm whale Mb to 740 s^{-1} for the mutant *His(E7)Val*. It is very interesting and unequivocal, from data shown in Fig. 1 and Table I, that the double mutant *His(E7)Val-Thr(E10)Arg* displays an intermediate behaviour ($k = 48$ s^{-1}).

A similar trend has been observed in the case of O_2. Thus, estimates of the rate constant for O_2 dissociation indicate that the mutant *His(E7)Val* releases O_2 with a very high rate constant ($k = 2.3 \times 10^4$ s^{-1}) [7]; next, the double mutant *His(E7)Val-Thr(E10)Arg* displays a considerably smaller value of k ($= 7.0 \times 10^2$ s^{-1}), though still higher than in the wild-type sperm whale Mb ($k = 10$ s^{-1}) [10], as well as in *A. limacina* Mb ($k = 7.0 \times 10^1$ s^{-1}) [10]. Rohlfs et al. [7] have reported that the mutant *His(E7)Val* has a very high value of the CO combination rate constant ($k' = 7.0 \times 10^6$ $M^{-1} \cdot s^{-1}$). We have confirmed this observation and found that the double mutant *His(E7)Val-Thr(E10)Arg* displays a similar value of k' ($= 1.8 \times 10^7$ $M^{-1} \cdot s^{-1}$).

These new results have been rationalized by reference to the structure of the N_3^- derivative of ferric sperm whale Mb [18], starting from the hypothesis of Bolognesi et al. [8], discussed above. The stabilization of the bound ligand observed in the double mutant is documented by the values of the N_3^- dissociation rate constant (see Fig. 1 and Table I). The kinetic effects reported in Table I are interpreted assuming that Arg(E10) is long and flexible enough to reach the distal pocket, stabilizing the heme bound ligand by hydrogen bonding (see Fig. 2). As suggested from values of the N_3^- dissociation rate constants for the *His(E7)Val-Thr(E10)Arg* double mutant ($k = 4.8 \times 10^1$ s^{-1}) and *A. limacina* Mb ($k = 7.5 \times 10^2$ s^{-1}) [14], the role of Arg(E10) in the stabilization mechanism is more effective in the former case. This may be accounted for on the basis of the substantially different binding mode of N_3^- in the distal pocket of *A. limacina* (M. Bolognesi, personal communication) and sperm whale [18] Mb's, which allows different degrees of stabilization by Arg(E10). In fact, in sperm whale Mb, N_3^- is fully buried in the heme crevice and points towards residue Leu(B10); on the other hand, in *A. limacina* Mb the ligand is oriented towards the outer part of the heme crevice and hydrogen bonded to Arg(E10) at the protein/solvent interface.

It is also worthwhile to discuss the effect of the substitution Thr(E10)Arg on the O_2 dissociation rate constant (see above). Model building on wild-type and mutant sperm whale Mb's indicates that the presence of residue Arg(CD3) can interfere with the ligand stabilization mechanism proposed by Bolognesi et al. [8]. Both Arg(CD3) and Arg(E10) can extend their side chains well inside the heme distal pocket, affecting their mutual positions by electrostatic repulsion. On the other hand, anionic ligands might partly compensate for the Arg(CD3):Arg(E10) unfavourable interaction. A similar phenomenon does not occur in *A. limacina*

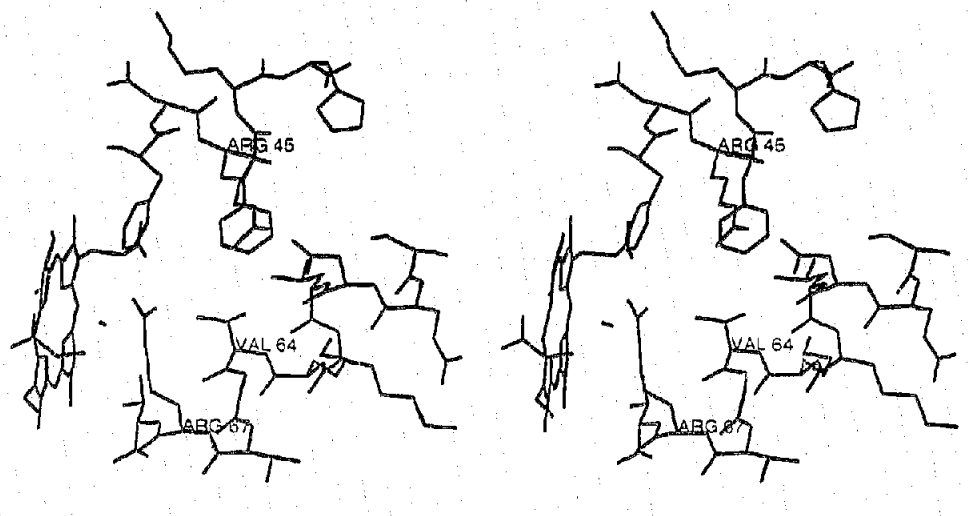

Fig. 2. Stereo view of the distal site environment, seen from the solvent side of the *His(E7)Val-Thr(E10)Arg* double mutant of sperm whale Mb as model-built on the crystal structure of the sperm whale Mb:N_3^- adduct, refined at 1.9 Å resolution (M. Bolognesi, personal communication). The E helix is on the right end side of the picture; the N_3^- molecule is shown between the heme and the side chain of Arg^{67} (E10).

Mb, where position CD3 is occupied by Asp, which makes a salt bridge with Lys(E3). On these grounds we expect that additional mutations, initially involving replacement of Arg(CD3) in the *His(E7)Val-Thr-(E10)Arg* double mutant, may lead to a more complete stabilization of the distal ligand; this hypothesis being tested by additional experiments. Finally it is of great interest that N_3^- binding is shown to be a remarkably sensitive and useful probe for investigating the control of ligand dynamics by the structure of the distal pocket.

Acknowledgements: The investigation was partially supported by grants from MURST and CNR (Progetto speciale 89.04618.14 'Metaloproteine') (Italy) and National Institutes of Health grants PHS R01 GM33775 and PHS R01 GM31756 (USA). C.T.A. is a fellow of I.B.P.E. (Naples, Italy).

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