Formate binding to ferric wild type and mutant myoglobins Thermodynamic and X-ray crystallographic study

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Abstract The X-ray crystal structure of the formate derivative of ferric loggerhead sea turtle (*Caretta caretta*) Mb has been determined at 2.0 Å resolution ($R = 0.164$) by difference Fourier techniques. Formate, sitting in the central part of the heme distal site, is coordinated to the heme iron as unidentate ligand, through the O1 oxygen atom, and is hydrogen bonded to the distal His^{64}(E7) NE2 atom through O2. Thermodynamics for formate binding to ferric loggerhead sea turtle Mb, sperm whale Mb, *Aplysia limacina* Mb, as well as to the VR and VRS mutants of sperm whale Mb were obtained between pH 4.5 and 8.5, at 20.0°C. These results, representing the first structure of a ferric hemoprotein:formate complex solved by X-ray crystallography, outline the role of amino acid residues at positions E7, F8 and E10 in modulating ligand binding properties of oxygen carrying proteins.

Key words: *Aplysia limacina* myoglobin; Loggerhead sea turtle (*Caretta caretta*) myoglobin; Sperm whale (*Physeter catodon*) myoglobin; Wild type and mutant myoglobin; X-ray crystal structure (of the ferric loggerhead sea turtle myoglobin:formate complex); Formate binding; Thermodynamics

1. Introduction

Oxygen carrying heme proteins share a highly conserved three-dimensional structure, despite low levels of sequence homology [1]. The heme-iron ligand stabilization mechanism in heme proteins has been studied, both for ligands of the ferrous and of the ferric form [2]. In the course of our studies on the reactivity of wild type and mutant monomeric globins, we have become interested in the functional and structural properties of heme proteins lacking the highly conserved distal residue His(E7), known to stabilize the heme iron-bound ligand through hydrogen bonding [3–9]. In *A. limacina* Mb, absence of the distal His(E7) (substituted by Val) is partially compensated by the presence of an arginyl residue at position E10, which upon ligand binding has been shown to fold into the distal site triggering the formation of extended polar interactions which stabilize the heme iron-bound ligand [5]. Also in sperm whale Mb mutants, removal of His(E7) can be partially compensated by introduction of an arginyl residue at position E10 [4,6].

In order to shed more light on the ligand recognition processes operating in wild type and mutant Mb's, the X-ray crystal structure of the formate derivative of ferric loggerhead sea turtle (*Caretta caretta*) Mb has been determined at 2.0 Å resolution. Moreover, thermodynamics for formate binding to ferric loggerhead sea turtle Mb, sperm whale Mb, *A. limacina* Mb as well as the VR and VRS mutants of sperm whale Mb were obtained between pH 4.5 and 8.5, at 20.0°C. These results outline the role of the amino acid residues at positions E7, F8 and E10 in modulating ligand binding properties of oxygen carrying proteins.

2. Materials and methods

Ferric *A. limacina* Mb and loggerhead sea turtle Mb were prepared as previously reported [5,10]. Wild type as well as VR and VRS mutants of sperm whale Mb were prepared as detailed elsewhere [4,6]. Formate, MES and TES were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All the other products were from Merck AG (Darmstadt, Germany). All chemicals were of analytical grade and used without further purification.

Crystals of ferric loggerhead sea turtle Mb were grown from ammonium sulfate solutions as previously described [10]. Preparation of the formate derivative was achieved by soaking of the protein crystals in their mother liquor solution containing saturating levels of formate, at pH 5.6 (0.05 M acetate buffer) and 25.0°C, for 2 h. Diffraction data to 2.0 Å resolution were collected on a Rigaku R-axis II imaging plate system, using CuKα radiation. A total of 25,108 reflections were reduced to 10,417 independent structure factors using Molecular Structure Corporation (The Woodlands, TX, USA) proprietary software ($R_{merge} = 0.045$, completeness 95%, in the 10.0 to 2.0 Å resolution shell).

The structure analysis was initiated by rigid body refinement of the unliganded ferric loggerhead sea turtle Mb atomic coordinates [8] against the ferric protein:formate complex structure factors; after 7 TNT cycles [11], the *R*-factor dropped from 0.34 to 0.26 (as calculated in the 10.0 to 2.0 Å resolution range). Subsequently, inspection of the $2F_d - F_n$ and $F_d - F_n$ difference electron density maps, whose phases were calculated on the basis of the unliganded ferric loggerhead sea turtle Mb refined structure [8] (F_d is the amplitude of the observed structure factor for the ferric loggerhead sea turtle Mb:fornate complex, and F_n is the amplitude of the unliganded ferric protein structure factor), showed unambiguously the heme-iron bound formate mole-

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Abbreviations: Mb, myoglobin; *A. limacina*, *Aplysia limacina*; VR, His(E7)→Val and Thr(E10)→Arg sperm whale Mb mutant; VRS, His(E7)→Val, Thr(E10)→Arg and Arg(CD3)→Ser sperm whale Mb mutant; MES, 2-[*N*-morpholino]ethanesulphonic acid; TES, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulphonic acid; r.m.s., root mean square.

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cule. Restrained crystallographic refinement of the structure (omitting the formate contribution from phase calculation) was then performed, alternating TNT cycles [11] with map inspection using FRODO [12]. When the *R*-factor reached 0.21, the triatomic formate molecule contribution was introduced in all calculations, and the refinement continued for 6 cycles along the same lines. The refined model contains 1273 protein atoms, 110 solvent molecules, and the formate molecule; the corresponding *R*-factor is 0.164 (for the data in the 10.0 to 2.0 Å resolution range) with ideal protein stereochemical parameters (r.m.s. deviation for bond lengths is 0.015 Å, and for angles is 2.25°).

Values of the apparent association equilibrium costant (K_a) for formate binding to ferric loggerhead sea turtle Mb, sperm whale Mb, *A. limacina* Mb, as well as the VR and VRS mutants of sperm whale Mb were determined spectrophotometrically between pH 4.5 and 8.5, at 20.0°C [13]. Values of the intrinsic association equilibrium constant (K_0) were calculated from values of K_a, according to the following equation [13]:

$$K_0 = K_a \cdot \{[H^+] + K_p\}/[H^+] \quad (1)$$

where K_p is the proton dissociation equilibrium constant for the reversible 'acid-alkaline' transition of the ferric protein. Values of pK_p for loggerhead sea turtle Mb, sperm whale Mb, *A. limacina* Mb, as well as for VR and VRS mutants of sperm whale Mb, at 20.0°C, are 8.5 [8], 8.95 [14], 7.6 [14], 8.1 [6] and 8.4 (present study), respectively.

3. Results and discussion

The ferric loggerhead sea turtle Mb:formate complex refined structure is shown in Fig. 1, relative to the protein heme distal site where the ligand is bound. Formate is coordinated to the heme iron, as unidentate ligand, through the *anti* lone-pair orbital of the oxygen atom O1, at a coordination distance of 2.21 Å. The formate 'boomerang-shaped' molecule is roughly perpendicular to the heme plane, slightly tilted towards the inner part of the distal region of the heme pocket (the Fe-O1-C angle is 124°), and approximately contained in a plane passing through the CHA and CHC methinic bridges. As a result of this orientation, the formate O2 atom is hydrogen bonded to the distal His^{64}(E7) NE2 atom (2.72 Å).

Although *anti* bonding is less frequently observed than *syn* in metal ion-carboxylate coordination, the presence of the distal His^{64}(E7) side chain and of the hydrogen bond between the O2 atom of formate and the NE2 atom of His^{64}(E7) favour such a structural organization [15,16].

The formate orientation observed in the ferric loggerhead sea turtle Mb:ligand complex is strongly reminiscent of that of acetate observed in the ferric leghemoglobin:ligand adduct [17]. Indeed, the acetate carboxylate group is in a orientation nearly identical to that of formate, the methyl group pointing towards the inner part of the distal region of the heme pocket.

The heme iron is contained in the porphyrin plane (0.02 Å out of the heme pyrrole plane, in the proximal direction), the coordination bond to the proximal His^{93}(F8) being 2.31 Å long.

A least square fit of the unliganded ferric loggerhead sea turtle Mb [8] and of its formate derivative three-dimensional structures yields a value of 0.61 Å for the r.m.s. deviation between the two complete proteins. Such a large deviation is the result of a local structure displacement occurring in the CD-D Ala^{44}-Lys^{56} region, where deviations up to 2.2 Å between the two structures can be observed. This finding is in agreement with a significant unit cell contraction (1.2 Å along the *c* and 0.6 Å along the *a* axes, respectively) observed in the ferric loggerhead sea turtle Mb crystals after soaking in the formate containing mother liquor. Such a structural rearrangement in the CD-D region of the molecule (which is largely exposed to the solvent in the crystalline lattice) may result from the ferric formate derivative soaking conditions, which differ substantially from those of the unliganded ferric protein crystals. Nevertheless, careful inspection of the electron density maps does not allow to identify aspecific binding sites for formate, or ionizable residues, which could trigger the local conformational readjustment observed.

Over the whole pH range explored (i.e. between pH 4.5 and 8.5), the affinity of formate for the VR and VRS mutants of sperm whale Mb and for *A. limacina* Mb is lower than that observed for ligand association to loggerhead sea turtle Mb and sperm whale Mb (see Fig. 2). These results outline the role exerted by the distal His^{64}(E7) residue in the strong stabilization of the loggerhead sea turtle Mb: and sperm whale Mb:formate adducts (see Fig. 1). In this respect, Arg(E10) has been also observed to exert a role in the weak stabilization of heme iron ligands in the VR and VRS mutants of sperm whale Mb as well as in *A. limacina* Mb, all displaying a valyl residue at position E7 [4–6]. In agreement with Travaglini Allocatelli et al. [6], the presence of a seryl residue at position CD3 in the VRS mutant of sperm whale Mb, instead of Lys in loggerhead sea turtle Mb, Asp in *A. limacina* Mb, and Arg in the VR mutant of sperm whale Mb as well as in the wild type hemoprotein, does not affect significantly formate binding properties (see Fig. 2).

The intrinsic affinity for formate binding to the monomeric hemoproteins considered here increases as the pH is lowered (see Fig. 2). The pH dependence of logK_0 for ligand binding to the loggerhead sea turtle Mb and sperm whale Mb reflects the alkaline p*K*-shift of a single group from 6.4, in the ligand-free Mb, to 6.8, in the formate derivative (see Fig. 2). On the other hand, formate binding to the VR and VRS mutants of sperm

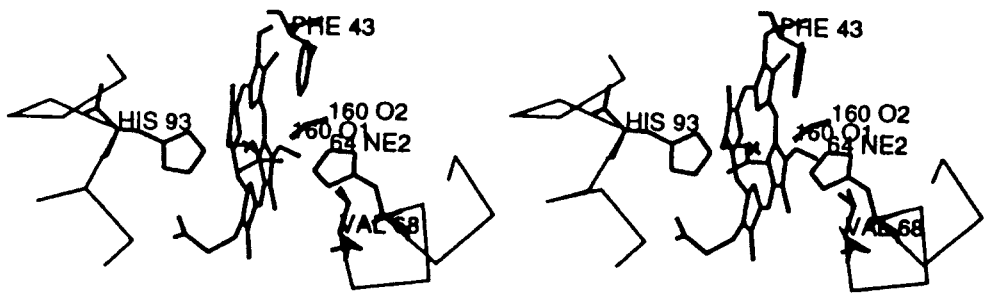

Fig. 1. Stereoscopic view of the distal site of the formate derivative of ferric loggerhead sea turtle Mb, as seen from the solvent side. The E helix is on the right. Residues Phe^{43}(CD1), His^{64}(E7), Val^{68}(E11) and His^{93}(F8) are highlighted. The formate molecule has the identification number 160

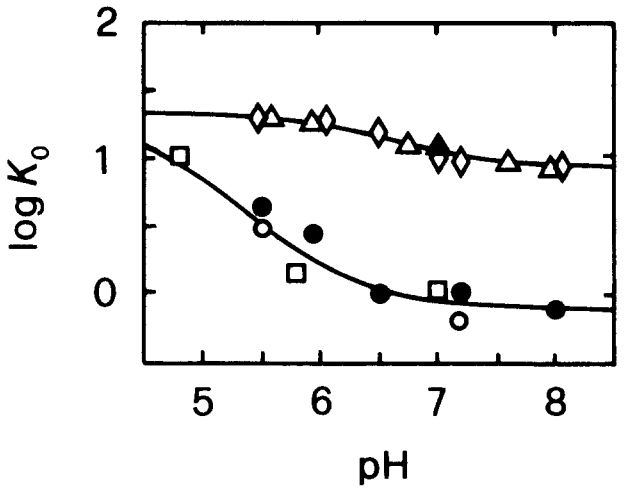

Fig. 2. Effect of pH on the intrinsic association equilibrium constant (K_0; M^{-1}) for formate binding to loggerhead sea turtle Mb (△), sperm whale Mb (◇), VR (●) and VRS (○) mutants of sperm whale Mb and *A. limacina* Mb (□). The solid curves were calculated according to the following equation [13]:

$$\log K_0 = C - \log\left\{\frac{\{[H^+]+K_{UNL}\}}{\{[H^+]+K_{LIG}\}}\right\} - \log\left\{\frac{K_{LIG}}{K_{UNL}}\right\}$$

where C is a constant that corresponds to the alkaline asymptote of $\log K_0$, and pK_{UNL} and pK_{LIG} are the pK values of the apparent proton dissociation equilibrium constant for the ligand-free (pK_{UNL}) and the ligand-bound (pK_{LIG}) hemoprotein, respectively. The solid curves were calculated with the following sets of parameters: wild type sperm whale Mb/ and loggerhead sea turtle/formate systems – C = + 0.94, pK_{UNL} = 6.4, and pK_{LIG} = 6.8; VR and VRS mutants of sperm whale Mb/ and *A. limacina* Mb/formate systems – C = – 0.10, pK_{UNL} = 4.7, and pK_{LIG} = 6.1. The pH profile was explored using the following 0.2 M buffer systems: MES/NaOH (pH 4.5 to 6.0), phosphate (pH 6.0 to 7.5) and TES/H_3PO_4 (pH 7.5 to 8.5). For further details, see text.

whale Mb and *A. limacina* Mb reflects the alkaline pK-shift of a single group from 4.7, in the ligand-free Mb, to 6.1, in the ligand-bound hemoprotein (see Fig. 2). The pK values calculated from the Bohr effect for formate binding to the ferric derivative of the hemoproteins considered are in excellent agreement with those evaluated from the pH dependence of their spectral properties [3,13]. This observation suggests that a single proton may affect both equilibria and spectroscopic properties of the ferric derivative of these hemoproteins.

The pH dependence of both spectroscopic and functional properties of sperm whale Mb and loggerhead sea turtle Mb probably reflects the acid-base equilibrium of the NE2 atom of the distal His^{64}(E7) residue [3]. On the other hand, in *A. limacina* Mb and in the VR and VRS mutants of sperm whale Mb, the effect of pH on both spectroscopic and functional properties may reflect the weakening of the proximal HisF8-Fe atom bond, which could result from an interaction between the iron atom and the protonated HisF8 imidazole ring [13,18].

Finally, as shown in Fig. 2, the VR and VRS mutants of sperm whale Mb as well as *A. limacina* Mb, all lacking the distal histidine, display an enhanced and acid-shifted Bohr effect for anionic ligand binding with respect to the loggerhead sea turtle Mb and wild type sperm whale Mb. Such finding suggests that mutations at positions E7 (His→Val) and E10 (Thr→Arg) induce subtle structural changes in the heme pocket which affect pK values of amino acid residues modulating spectroscopic and ligand binding properties of the monomeric hemoproteins considered here. In this respect, it may be recalled that in the VR and VRS mutants of sperm whale Mb as well as in *A. limacina* Mb, the E helix is closer to the heme iron with respect to wild type sperm whale Mb to minimize the vacancy resulting from the His(E7)→Val substitution [5,19].

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