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Crystal structure of a distal site double mutant of sperm whale myoglobin at 1.6 Å resolution

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The three-dimensional structure of sperm whale myoglobin His[64](E7)→Val,Thr[67](E10)→Arg double mutant has been studied by X-ray crystallography at 1.6 Å resolution, and refined to a crystallographic *R*-factor of 0.197. The Arg[67](E10) side chain is extended in the direction of the ligand binding site, and its NH1 atom is at a distance of 3.11 Å from the NH1 atom of Arg[45](CD3), which is also pointing towards the distal site. Both are kept in this position by hydrogen bonding and electrostatic interactions with a solvent sulfate ion, located amongst the two, on the protein surface. No liganded water molecule is present at the sixth coordination position of the Fe(III) heme.

Protein engineering; Myoglobin mutant; Ligand binding

1. INTRODUCTION

Heterologous expression of heme proteins in *E. coli* has provided, through site-directed mutagenesis, a powerful tool to define the extent to which individual side chains contribute to ligand recognition and stabilization. Along these lines, a large body of functional data on equilibrium and kinetic processes concerning several different hemoprotein mutants is available. Nevertheless, complete characterization of the engineered proteins requires thorough structural analysis in order to: (i) correlate crystal structures with ^{1}H NMR information and with dynamics; (ii) investigate subtle structural effects in the discrimination of ligands (e.g. CO vs. O_2); and (iii) shed more light on the evolutionary significance of alternative ligand stabilization mechanisms and molecular recognition in myoglobins from different species.

In this sense a paradigmatic case is that of the 'distal' histidine, His[64](E7) in sperm-whale myoglobin (Mb), which has been proposed to act as a 'swinging door' residue, gating the entry of ligands (such as CO, imidazole and isocyanides) in the distal site [1–6]. This hypothesis has been proven not only by functional studies on site-directed mutants [3], but also by structural investigations [4–6], which show His[64](E7) in different conformations swinging in and out of the heme pocket. Along the same lines, in *Aplysia limacina* Mb, which lacks His(E7) (substituted by Val[63](E7) at the distal site), the role exerted by Arg[66](E10) in ligand stabilization has been detailed both by crystallographic [7] and by ^{1}H-NMR [8] studies.

In an attempt to reproduce an *Aplysia*-like ligand stabilization mechanism, Cutruzzolà et al. [9] have prepared a sperm whale Mb double mutant His[64](E7)→Val,Thr[67](E10)→Arg (VR mutant). In the case of ligands of the ferric form (such as azide), the presence of an arginyl residue at position E10 is sufficient to recover affinity and control of kinetics lost in the His[64](E7)→Val mutant. To further support this result, ^{1}H NMR measurements on the cyanide ferric derivative of this double mutant show a hydrogen bonding interaction between Arg[67](E10) and the iron co-ordinated cyanide ion [10].

As a conclusive step towards complete structural characterization, we present here the 1.6 Å-refined X-ray crystal structure of the ferric form of the VR double mutant. Details of the structural organization of the mutated distal residues in relation to the ligand binding properties in this molecule are discussed below.

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Abbreviations: NMR, nuclear magnetic resonance; V mutant, His[64](E7)→Val sperm whale myoglobin single mutant; VR mutant, His[64](E7)→Val,Thr[67](E10)→Arg sperm whale myoglobin double mutant; Mb, myoglobin; rms, root mean square; R_{sym}, merging *R*-factor between symmetry related reflections:

$$R_{sym} = \sum_h \sum_j | I_{h,j} - \langle I \rangle_h | / \sum_h \sum_j I_{h,j},$$

where I_h is the observed intensity of the *j*-th measurement of reflection *h*, and $\langle I_h \rangle$ is its mean value. Amino acid residues have been identified by their three-letter codes, sequence number and topological position [1] within the conventional globin fold.

2. MATERIALS AND METHODS

Protein purification was performed as previously described [9]. Purity was checked spectrophotometrically, measuring the 395/280 nm absorbance ratio, and by SDS-PAGE. Crystal growth was performed at room temperature using the hanging drop vapor diffusion technique as described by Phillips et al. [11]. Drops were obtained by mixing 5 μl of 1 mM protein solution with 5 μl of 2.6–2.8 M ammonium sulfate solutions containing 20 mM Tris-HCl buffer, 1 mM EDTA, at pH 9.0. The crystals obtained belong to the hexagonal space group P6, with unit cell parameters $a = b = 91.30$ Å $c = 45.86$ Å; they are isomorphous with those obtained by Phillips et al. [11] for the recombinant sperm whale Mb. For data collection crystals were soaked in 20 mM Tris-HCl buffer, 2.8 M ammonium sulfate, 1 mM EDTA, at pH 7.0. Diffraction data to a limiting spacing of 1.6 Å were collected on a Rigaku R-axis II image plate detector system during a test visit at Molecular Structure Corp. (Houston, Texas, USA), using one crystal. Images were processed using MSC's proprietary software package, based on the oscillation film processing system MOSFLM [12]. Altogether 62,341 observed reflections were reduced to 15,938 independent structure factors, with a R_{sym} factor value of 0.052, based on intensities (53% of the theoretical reflections in the 15.0–1.6 Å resolution shell).

The starting atomic coordinates, on which phases and difference Fourier maps were firstly calculated, were those of the wild-type protein [11], from which His^{64}(E7), Thr^{67}(E10) and the distal ligand were omitted. A starting R-factor of 0.26 was calculated, in the 15.0–1.6 Å resolution shell. After inspection of the initial 'omit' Fourier map, which showed clear electron density for the mutated side chains, restrained crystallographic refinement was carried out, alternating refinement and model inspection/correction cycles using the TNT and FRODO software packages, respectively [13,14]. The crystallographic R-factor, after 3 cycles, was 0.197 in the 15.0–1.6 Å resolution range, with a protein stereochemistry close to ideal (rms deviation of bond lengths is 0.016 Å from ideality, and the corresponding figure for bond angles is 2.06°). Atomic coordinates of the refined model have been deposited with the Brookhaven Protein Data Bank, Upton (NY, USA), from which copies are available [15].

The affinity constant for azide was determined spectrophotometrically in 0.1 M sodium phosphate buffer, pH 7.0, 20°C, using a Cary 210 spectrophotometer (Varian), following Giacometti et al. [16]. All the reagents used were of analytical grade.

3. RESULTS AND DISCUSSION

The overall structure of the VR mutant, as expected, conforms closely to that of the wild-type protein [11]. Therefore discussion of its structure is limited to the distal site region, where amino acid substitutions have been introduced. Fig. 1 shows a close view of the distal site, as seen from the solvent space outside the protein. As became clear from the first stage of the refinement, Arg^{67}(E10) is extended in the direction of the ligand binding site, close to Val^{64}(E7). Moreover, the NH1 atom of Arg^{67}(E10) is 3.11 Å from the NH1 atom of Arg^{45}(CD3) which is also pointing towards the distal site. Both arginyl residues are kept in this position by hydrogen bonding and electrostatic interactions with a solvent sulfate ion, located between the two guanidino groups. In this orientation both residues are at the very entrance of the distal pocket, 50.1% and 44.8% solvent accessible, respectively. The 'nested' sulfate ion is 13.5 Å away from the heme iron, in a protein surface location; its molecular surface is 113 Å^2 solvent accessible [17]. As can be seen from Fig. 1 the Arg^{45}(CD3)–Arg^{67}(E10) pair, together with the sulfate anion, virtually acts as a physical barrier between the distal ligand binding site and the outer solvent space. Polar interactions observed in this region are listed in Table I.

Inspection of the electron density maps shows that no liganded water molecule is present at the iron 6th coordination position, in accordance with what has been observed for the distal site of *A. limacina* Mb [7,18]. Next, a strong electron density peak in the neighborhood of residue Glu^{52} is clearly interpretable as a sulfate ion, hydrogen bonded to the peptide N atom of residue 52 (2.93 Å), as also observed in the wild-type protein structure [11].

The crystal structure of the VR double mutant reported here conforms to expectations, based on functional studies, in two ways. When His(E7) is replaced by Val (and other apolar residues), no water molecule is bound at the 6th coordination position of the Fe(III), as shown by optical and ^{1}H NMR spectroscopy [10,19]. Moreover, simple conformational readjustements can indeed bring the side chain of Arg^{67}(E10) in a position

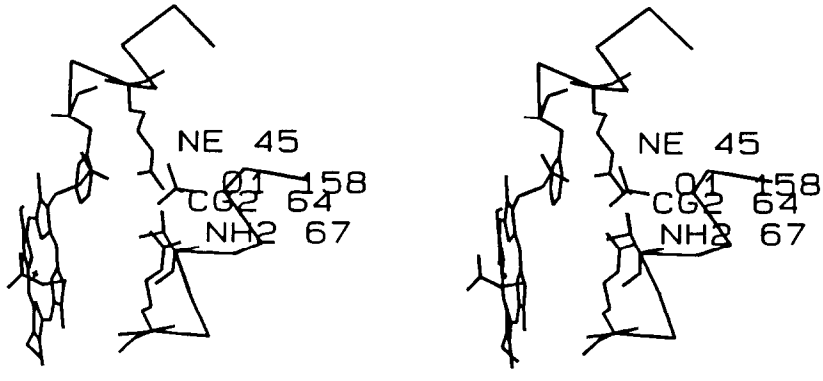

Fig. 1. Stereo view of the heme and residues surrounding the distal site in the crystal structure of sperm whale Mb VR mutant, as seen from the solvent side. The surface sulfate ion located between Arg^{45} (CD3) and Arg^{67} (E10) is labeled with identification number 158. Cα traces have been indicated for part of the CD region and of the E helix.

Table I

Polar interactions observed in the distal site region

Atom 1	Atom 2	Distance (Å)
O2D Heme	NE Arg^{45}	3.10
O2D Heme	NH2 Arg^{45}	2.64
NH1 Arg^{45}	NH1 Arg^{67}	3.11
NH1 Arg^{67}	O2 Sulfate 158	2.99
NH2 Arg^{67}	O2 Sulfate 158	2.80
NH2 Arg^{67}	O1 Sulfate 158	3.12

suitable for ligand stabilization through hydrogen bonding and/or electrostatic interaction, as in the case of *A. limacina* Mb [7,18].

The presence of a sulfate ion, bridging between Arg^{45}(CD3) and Arg^{67}(E10), on the protein surface, might be suggestive of an electrostatically stabilized distal gate, capable of affecting ligand association processes in the VR mutant. In order to test the possibility that also in aqueous solution a sulfate ion might have a role in sequestering the Arg(E10) side chain out of the distal pocket, we have determined the affinity constants for azide binding in the presence and absence of 1 M ammonium sulfate. If the sulfate ion stabilized Arg^{67}(E10) outside the heme pocket also in solution the affinity of the VR mutant for N_3^- should be expected to decrease significantly upon addition of sulfate and eventually approach the value observed for the V mutant (which was shown to display a very low affinity for anionic ligands, Cutruzzolà et al. [9]). As shown in Fig. 2, such an effect is quite marginal, and present to a comparable extent in the wild-type protein, which displays Thr^{67} at the E10 position.

The limited effect of 1 M ammonium sulfate on the binding constants observed on VR and wild-type Mb's can be interpreted as a global ionic strength effect, independent from distal site determinants. No effect was observed on the V mutant, probably as a consequence of its intrinsic low affinity for azide. Inspection of molecular packing in the P6 crystalline form studied in the present paper indicates that position E10 falls in the

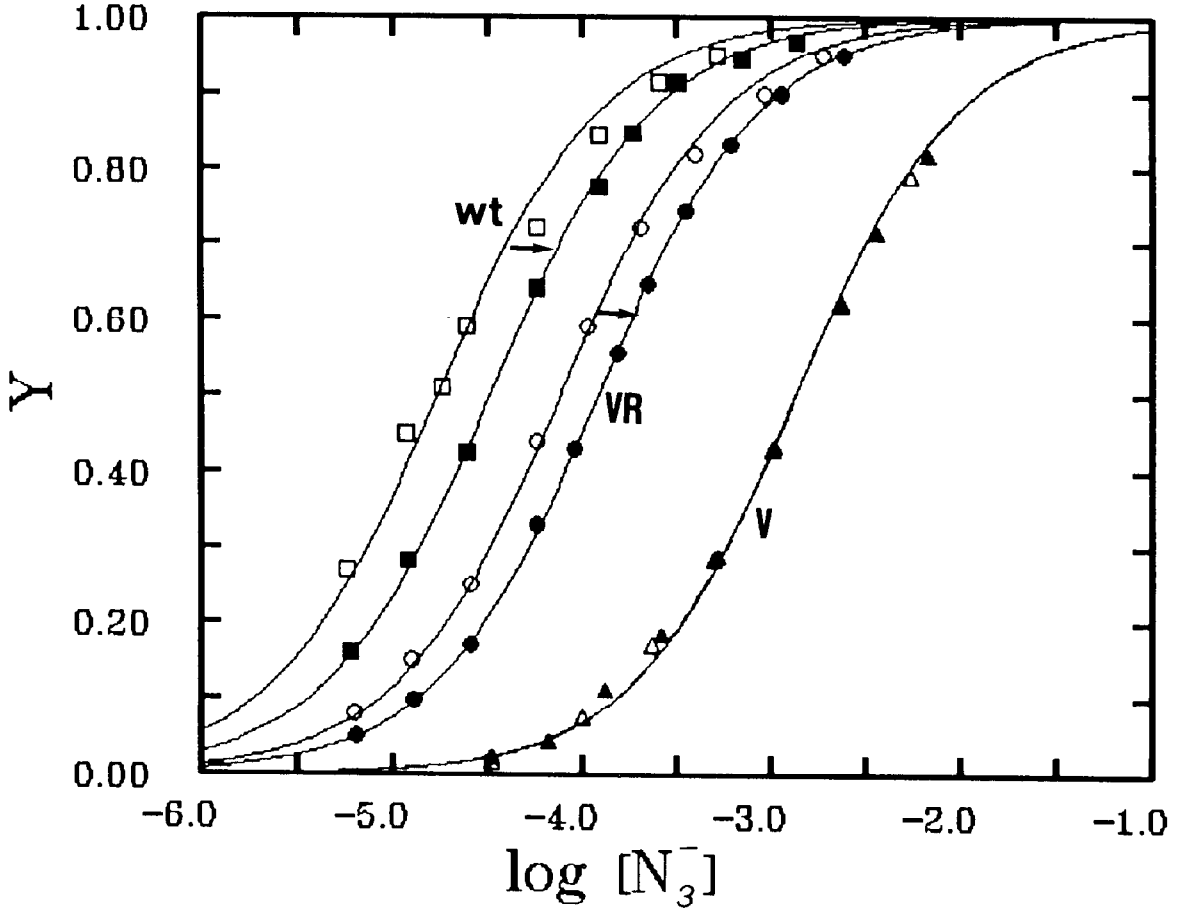

Fig. 2. Effect of ammonium sulfate on fractional saturation (Y) of sperm whale Mb for N_3^-. Results obtained with wild-type protein (squares), V mutant His^{64}(E7)→Val (triangles), and VR mutant His^{64}(E7)→Val,Thr^{67}(E10)→Arg (circles); open symbols indicate the values measured in the absence, and closed symbols in the presence of 1 M ammonium sulfate. Continuous lines refer to hyperbolic fits (Hill coefficent $n = 1$). Experimental conditions were 0.1 M phosphate buffer, pH 7.0, at 20 °C in the presence or absence of 1 M ammonium sulfate. Affinity constants (K_i) in the presence of sulfate decrease (as indicated by arrows) from 5.9×10^4 to 3.1×10^4 (M^{-1}) for wild-type, and from 1.25×10^4 to 7.6×10^3 (M^{-1}) for the VR mutant; in the case of the V mutant the K_i value, 6.7×10^2 (M^{-1}), is unaffected by the presence of sulfate.

crystal lattice 9.5 Å away from the same site in a symmetry related molecule. Moreover, in a 14 Å radius sphere (centered on the Arg^{67}(E10) guanidino group) four charged residues, Arg^{45}, Arg^{67}, Lys^{63} and Lys^{96} from neighboring molecules, are located. It is therefore conceivable that electrostatic contributions from adjacent molecules co-operate in favouring sulfate binding, in the crystalline VR mutant, at this molecular surface location.

Nevertheless, despite the peculiarity of the electrostatic field around site E10 in the crystal lattice, and in consideration of the marked differences in N_3^- affinities in the V and VR mutants, the data presented here underline the flexible structural and functional role of the Arg^{67}(E10) residue, which is fully compatible with the proposed mechanism of distal site ligand stabilization [7,10,18]. In accordance with this functional model, a very recent ^{1}H NMR study on the met-cyano derivative of Mb from the mollusc *Dolabella auricularia*, which contains an arginyl residue at position E10, has brought additional evidence supporting a ligand stabilization mechanism involving an Arg(E10) hydrogen bond [20].

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