Histidine 82 Influences Heme Orientational Isomerism in Sperm Whale Myoglobin. Long-Range Effect due to Mutation of a Conserved Residue

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Single amino acid substitutions in proteins can have unforeseen effects on structure,[1] folding kinetics,[2] and catalysis.[3] Our interest in the factors stabilizing the *b* hemoprotein myoglobin (Mb) from sperm whale with and without the prosthetic group[4-6] led us to probe the role of the conserved His-82 by a mutation. Here we show that heme affinity and the dynamics of polypeptide matrix–prosthetic group interactions are affected by a replacement that is not in the heme cavity.

His-82 is in the loop which connects the heme binding site helices E and F.[7] In holoMb, the imidazole group has a pK_a lower than 5.5.[6] The ring is less than 5% accessible to solvent and its center is located 11 Å from the nearest heme atom. His-82 (EF5) docks against the C-terminal helix (H helix) through an H-bond interaction with Asp-141 (H18). We selected substitution with a glutamine[8] to maintain H-bonding capability while preventing ionization.

Figure 1 contains NMR data collected on wild-type and mutant metaquoMb. Only heme protons resonate within the displayed region.[11] The weak peak marked m in the wild-type spectrum (Figure 1A) arises from a myoglobin isomer which differs from the crystallographic form by a 180° rotation of the heme group about the α,γ-meso axis.[12] The "reverse" isomer accounts for ca. 8% of the protein at equilibrium.[13,14] The mutant spectrum (Figure 1B) indicates that this equilibrium percentage is lowered by the change at position 82.

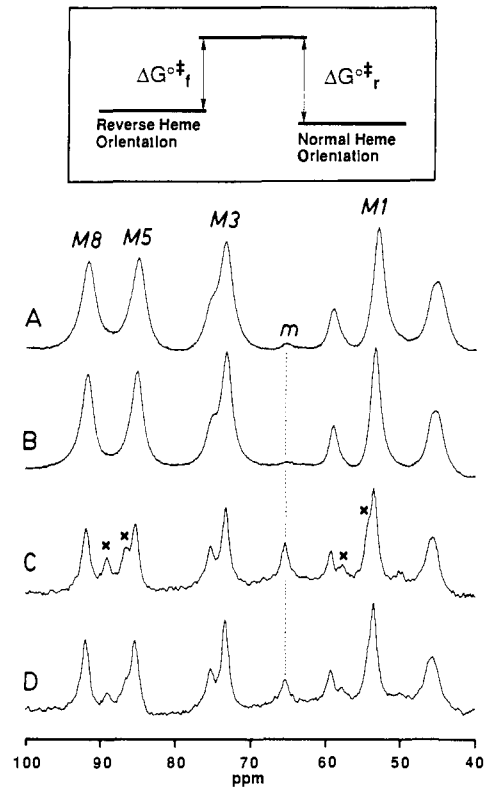

Figure 1. ^{1}H NMR spectra (500 MHz) of sperm whale metaquoMb collected with a 40.9-ms recycle time at 298 K, pH* (uncorrected for isotope effects) 7.5: (A) wild-type Mb at equilibrium; (B) His-82-Gln Mb at equilibrium; (C) reconstituted His-82-Gln Mb, after 8 min of equilibration; (D) reconstituted His-82-Gln Mb, after 34 min of equilibration. Reconstitution was accomplished by adding an equivalent amount of hemin in 0.1 M NaO^2H to 2.09 mM apo-Mb in ^2H_2O at pH* 6.7. The pH* was immediately adjusted to 7.5 with 0.1 M ^2HCl. A total of 1024 transients were acquired every 3 min to monitor the reorientation reaction. M1, M3, M5, and M8 mark the 1, 3, 5, and 8 heme methyl group resonances in the normal orientation, respectively;[12] m denotes a reverse isomer methyl signal. Crosses in spectrum C denote other heme signals from the reverse isomer. The inset shows the relative free energy diagram with the forward and reverse symbols used in the text.

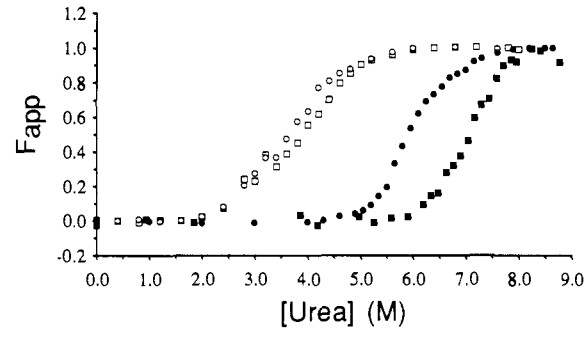

Figure 2. Denaturation of apo- and holoproteins at 298 K, pH 7.5. The apparent fractions of unfolded protein (Fapp)[23] for wild-type (□) and His-82-Gln (○) sperm whale apoMb were calculated from ellipticity measurements at 222 nm with 1 μM protein 10 mM HEPES buffer. Fapp values for wild-type (■) and His-82-Gln (●) sperm whale metaquoMb were calculated from absorbance measurements at 409 nm with 5 μM protein in 20 mM sodium phosphate buffer and 0.1 M KCl, after a 3-h equilibration period.

A reconstitution experiment was undertaken to support this observation. The reaction between hemin and apomyoglobin occurs on a millisecond time scale at neutral pH[15] and produces an equal mixture of the two heme orientational isomers.[16] The

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isomer concentrations then evolve to achieve thermodynamic proportions following a phenomenological first-order process:

$$\text{reverse} \underset{k_r}{\overset{k_f}{\rightleftharpoons}} \text{normal}$$

with $k_f/k_r = K_{eq}$ and $k_f + k_r = k_{obsd}$. In wild-type metaquoMb, reorientation is slowest at neutral pH, and the observed rate $^{wt}k_{obsd}$ is ca. 3.7×10^{-4} min^{-1} at pH 7.5.[14] If this rate is maintained in His-82-Gln Mb, the mutant reverse isomer should be detectable for days. The reorientation is illustrated in Figure 1: a spectrum collected after 8 min of equilibration (Figure 1C) exhibits partially resolved reverse isomer peaks at the same positions as in wild-type Mb. Simulation and integration of spectrum 1B determine the equilibrium proportion of the two forms in His-82-Gln Mb. The equilibrium constant $^{82}K_{eq}$ is ~20 compared to 11 in wild-type Mb. Thus, the His-82-Gln replacement destabilizes the minor form with respect to the major form by about 1.5 kJ mol^{-1} at neutral pH.

When the area of peak m is followed as a function of reaction time (as in Figure 1C,D) and the data are fit to a first-order equation, $^{82}k_{obsd} \approx 2.2 \times 10^{-2}$ min^{-1} is obtained; i.e., given $^{82}K_{eq}$, the rate constant k_f is accelerated by a factor of ~60. This implies a decrease in $\Delta G^{\circ}_f{}^{\ddagger}$ by ~11 kJ mol^{-1}.[17] The value is larger than justified by the destabilization of the minor form, and the mutation must therefore affect the reverse barrier height ($\Delta G^{\circ}_r{}^{\ddagger}$) as well.[18]

Barrier lowering can arise from destabilization of the ground states, stabilization of the transition state(s), or both. Destabilization of the ground state was tested by denaturation experiments with the assumption that the unfolded state is negligibly affected by the mutation at pH 7.5. The urea denaturation of wild-type and His-82-Gln Mb is illustrated in Figure 2. The curve for His-82-Gln holoMb is significantly displaced to lower denaturant concentrations compared to wild-type holoMb. Although non-two-state behavior prevents quantitation of the effect, ground-state destabilization does account for at least some of the acceleration of heme reorientation. Figure 2 also contains the denaturation curves for the apoproteins: apoMb is only slightly affected, if at all, by the mutation.

The His-82-Gln Mb results show that the imidazole ring at that position has a stabilizing role in native holoMb but not in native apoMb. In addition, although residue 82 is not in contact with the heme, it has a marked effect on the kinetics of heme reorientation and influences the ability of the protein to favor one isomer over the other. Heme reorientation has been investigated extensively with modified hemins.[14,24] However, protein matrix alterations have not been explored to determine the mechanism, which is most certainly complex as it reflects the conformational and chemical changes required to relocate the large heme group. F-helix unfolding may facilitate heme release[16,25] and is consistent with the view that the helix is conformationally labile even in the holoprotein.[26] It is possible that the EF-H interface must also separate for the heme to turn around. Model building shows that a glutamine cannot assume the same geometry with respect to Asp-141 as a histidine and may weaken the interactions between the two elements of structure. Further speculation on the molecular origin of the effect will require that more sites be targeted. Our observations suggest that the dynamics and thermodynamics of a designed protein could be adjusted without modifying functionally important prosthetic group and binding site residues.

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(18) k_{obsd} depends upon the sixth iron ligand[14] and may be correlated with the pI of the protein.[19] Basic titration of His-82-Gln Mb was monitored by visible spectroscopy at 542, 581, and 634 nm.[20] The mutation does not affect the transition from the metaquo to the methydroxy form, whose pK_a remains near 9.0.[21] Isoelectric focusing of wild-type and His-82-Gln Mb yields the same pattern of ferric and ferrous bands.[22]

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Characterization of a Dienol Intermediate in the 5-(Carboxymethyl)-2-oxo-3-hexene-1,6-dioate Decarboxylase Reaction

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5-(Carboxymethyl)-2-oxo-3-hexene-1,6-dioate decarboxylase (EC 4.1.1; COHED) from *Escherichia coli C* catalyzes the magnesium-dependent decarboxylation of **1** (Scheme I), a catabolite generated by the microbial degradation of (4-hydroxyphenyl)acetate.[1-3] The generally accepted mechanism for a β-decarboxylase that acts on an α-keto acid involves the intermediate formation of a metal-stabilized enol which ketonizes to the α-keto acid product.[4,5] In light of this mechanism, the COHED-catalyzed decarboxylation of **1** is particularly intriguing because the product is reportedly 2-hydroxy-2,4-heptadiene-1,7-dioate (**2**).[1,2,6] Because this conclusion rests solely on the observation and isolation of a compound with a λ_{max} at 276 nm from a reaction mixture containing **1** and COHED and no further characterization of this compound has been reported,[1,2,6] we initiated a rigorous investigation of the COHED reaction. We find that COHED generates a mixture of **2** and the β,γ-enone, 2-oxo-4-heptene-1,7-dioate (**3**; Scheme II). Moreover, incubation of **2** with COHED produces **3**. These results suggest that COHED catalyzes the decarboxylation of **1** to **3** through the intermediacy of **2** (Scheme II). To our knowledge, this is the first report of the characterization of a dienol as an intermediate in a metal-dependent decarboxylase reaction.

The substrate for COHED, **1**, is generated by the action of 5-(carboxymethyl)-2-hydroxymuconate isomerase (CHMI) on **4**.[7]

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(7) The isolation of **4**, CHMI, and COHED have been described.[1-3,6] In our hands, the final specific activity of COHED ranges from 160 to 250 units/mg of protein.

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