The D-Helix in Myoglobin and in the β Subunit of Hemoglobin Is Required for the Retention of Heme†

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Received April 13, 1995; Revised Manuscript Received May 18, 1995⊗

ABSTRACT: All globins consist of eight helices and interconnecting loops except α hemoglobin subunits which lack the D-helix due to deletion of five consecutive residues. Previous site-directed mutagenesis work suggested that this deletion is a neutral modification in hemoglobin with respect to equilibrium O_2 binding [Komiyama, N. H., Shih, T.-B., Looker, D., Tame, J., & Nagai, K. (1991) *Nature 352*, 349−351]. To examine the role of the D-helix in myoglobin, we have measured the O_2 and CO binding and hemin dissociation properties of recombinant sperm whale myoglobin mutants in which residues 51−55 have been deleted, Mb(−D), replaced by five alanines, Mb(Ala^{51-55}), and substituted with four alanines and a methionine, Mb($Ala^{51-54}Met^{55}$). Crystal structures of aquometMb(−D) and aquometMb($Ala^{51-54}Met^{55}$) were determined to 2.0 Å resolution and show that the conformation of the distal pocket is little affected by removal of the D-helix or mutations in this region. As a result, these mutations have little effect on O_2 and CO binding. Diffuse electron density is observed in the region between the C- and E-helices of Mb(−D), indicating a highly mobile or heterogeneous conformation in this portion of the tertiary structure. This flexibility provides an explanation for the 50-fold higher rate of hemin loss from Mb(−D) as compared to that from wild-type myoglobin. Hemin loss from Mb(Ala^{51-55}) is also rapid. In contrast, Mb($Ala^{51-54}Met^{55}$) shows a well-defined D-helix and has a rate of hemin loss identical to that of wild-type holoprotein. Thus, Met^{55} appears to play a key role in stabilizing the D-helix and CD corner in myoglobin. Deletion of the D-helix from β subunits in human hemoglobin also causes a marked increase in the rate of hemin loss, whereas there is no stabilizing effect when a D-helix is added to α subunits. The absence of a D-helix in the α subunits of chordate hemoglobins appears to be compensated by interactions with adjacent β subunits that stabilize the region between the C and E helices and prevent hemin dissociation.

Globular proteins consist of α helices and/or β strands which are connected by polypeptide loops. Sequence alignments of homologous proteins show that deletions and insertions of amino acids are accommodated in the loop regions. The D-helix and surrounding CD corner represent such a region in most of the globins of bacteria, plants, and animals (Figure 1; Fermi & Perutz, 1981). However, for unknown reasons the polypeptide chain between the C- and E-helices in α globins is five residues shorter, resulting in loss of helical secondary structure in this region of the protein (Kleinschmidt et al., 1987). Komiyama et al. (1991) examined the functional significance of the D-helix in β globins and its loss from α globins. They removed the D-helix from human β globin by deleting residues Thr^{50}-Pro-Asp-Ala-Val^{54}. This same set of residues was inserted into α globin starting at position 49. The corresponding mutants were designated β(−D) and α(+D), respectively, and two hemoglobin hybrids were made, α(wild type)β(−D) and α(+D)β(wild type). Wild type refers to subunits which are identical to the native proteins except for V1M substitutions to facilitate expression in *Escherichia coli* (Komiyama et al., 1991). Surprisingly, the O_2 equilibrium curves of the two mutants are similar to each other and to the wild-type control. No decrease in cooperativity or marked increase in O_2 affinity is observed. Komiyama et al. (1991) concluded that loss of the D-helix from α subunits was a functionally neutral mutation with respect to O_2 binding and assembly into a cooperative tetramer. However, this leaves unresolved the origin of the strong selective pressure to preserve a D-helix in the β subunits of vertebrate hemoglobins.

Like β subunits, all known chordate myoglobins have residues 50−55 which are normally assigned to the D-helix (Figure 1). However, both the functional and structural roles of the D-helix in myoglobin are unclear. Site-directed mutagenesis studies of residues in the CD corner of myoglobin have shown only modest effects on ligand binding (Lambright et al., 1988; Carver et al., 1991; Lai et al., 1995).

† Supported by U.S. Public Health Service Grants AR 40252 (G.N.P.), HL 31461 (K.N.), GM 35649 (J.S.O.), and HL 47020 (J.S.O.), Grants C-1142 (G.N.P.) and C-612 (J.S.O.) from the Robert A. Welch Foundation, Medical Research Council grants (K.N.), and the W. M. Keck Foundation. M.S.H. is a recipient of a predoctoral fellowship from the National Institutes of Health, Training Grant GM08280. M.B.B. is a recipient of a predoctoral fellowship from the National Science Foundation, Training Grant BIR 9256580.

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⊗ Abstract published in *Advance ACS Abstracts,* June 15, 1995.

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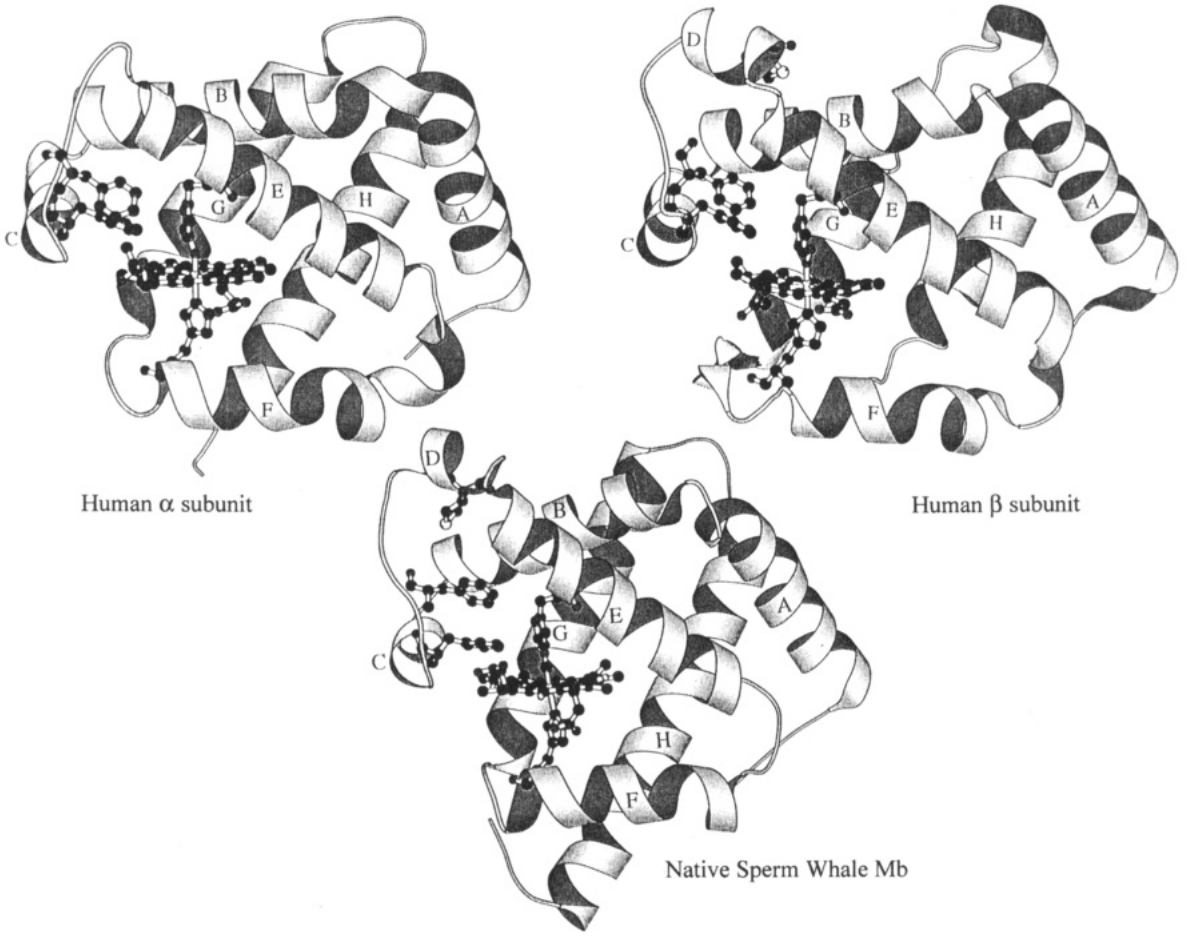

FIGURE 1: Ribbon drawings based on the three-dimensional X-ray structures of human α and β subunits and sperm whale myoglobin (Phillips, 1983; Quillin et al., 1994). The helices of each globin are labeled A through H, noting that α globin lacks a D-helix. The heme group and residues Phe(CD1), Phe(CD4), His(E7), and His(F8) are highlighted as ball and stick figures.

However, some of these substitutions cause marked increases in the rate of hemin dissociation (i.e., R45E or K45E at low pH, F43V, F43I, F46A, and F46V; Hargrove et al., 1994a). Since the D-helix connects the CD corner to the E-helix, it may play a role in stabilizing the globin structure to prevent hemin loss.

To test this idea, D-helix deletion and insertion mutants of sperm whale myoglobin were constructed in which residues Thr^{51}-Glu-Ala-Glu-Met^{55} were removed, Mb(−D), replaced with five alanines, Mb(Ala$^{51-55}$), and substituted with four alanines and one methionine, Mb(Ala$^{51-54}$Met^{55}). The Met^{55} residue is highly conserved in the D-helices of myoglobins.[1] Its large apolar side chain forms the core of a hydrophobic pocket directly under the D-helix, between the B-helix, the CD corner, and the beginning of the E-helix, and may be essential for the structural integrity of the D-helix (Figures 1 and 3). The structures of the ferric forms of Mb(−D) and Mb(Ala$^{51-54}$Met^{55}) were determined by X-ray crystallography, and the ligand binding, autooxidation, and hemin dissociation rate constants of all three mutants were measured. The D-helix mutations produce dramatic effects on the rates of hemin loss from myoglobin. These results led us to examine the hemin dissociation properties of the D-helix mutants of human hemoglobin that were originally constructed by Komiyama et al. (1991).

METHODS AND MATERIALS

Preparation of Recombinant Myoglobin and Hemoglobin. The original wild-type sperm whale myoglobin gene was constructed by Springer et al. (1989) and placed into the pEMBL19 vector for oligonucleotide-directed mutagenesis techniques (Carver et al., 1992). The sperm whale myoglobin D-helix deletion and insertion mutants were constructed at Rice University by site-directed oligonucleotide mutagenesis (Amersham Sculptor Mutagenesis kit). A long oligonucleotide was synthesized containing the complementary sequences for the regions before and after the code for residues 51−55. This was annealed to the wild-type gene, and the remaining steps were carried out as described in the Amersham kit. The recombinant myoglobins were expressed in TB-1 *E. coli* and purified as specified by Springer and Sligar (1987) and Carver et al. (1992).

The hemoglobin mutants, α(+D)β(wt) and α(wt)β(−D), were constructed by Komiyama et al. (1991) using cassette mutagenesis of the recombinant hemoglobin gene pSGE0.0E4. The pSGE0.0E4 vector contains one α subunit cistron and

[1] A listing of known myoglobin sequences was obtained using the EuGene & SAM software package developed by the Molecular Biology Information Resource, Department of Cell Biology, Baylor College of Medicine, Houston, TX, and the Protein Sequence Data Bank from the National Biochemical Research Foundation, Washington, DC. The alphanumeric codes (e.g., CD4, D5) refer to the positions of the residue within the helices and loops of the myoglobin folding pattern (Dickerson & Geis, 1983). For example, CD4 refers to the fourth residue at the CD corner (the loop motif between helices C and D) while D5 refers to the fifth residue in the D-helix. In *E. coli* recombinant myoglobins, amino acids at CD4 and D5 are still numbered as 46 and 55, respectively, for the purpose of consistency even through posttranslational processing in *E. coli* fails to cleave away the methionine at the N-terminus.

Table 1: O_2 Binding, Hemin Loss, and Autooxidation Parameters for D-Helix Mutants of Sperm Whale Myoglobin[a]

Mb mutant	k'_{O_2} (μM^{-1} s^{-1})	k_{O_2} (s^{-1})	K_{O_2} (μM^{-1})	k'_{CO} (μM^{-1} s^{-1})	k_{CO} (s^{-1})	K_{CO} (μM^{-1})	k_{-H} (h^{-1})	k_{ox} (h^{-1})
wild type	17	18	0.95	0.49	0.020	25	0.6	1.2
−D(51−55)	13	31	0.42	0.45	0.030	15	46	6.0
Ala^{51-55}	19	17	1.1	0.77	0.021	37	14	3.5
$Ala^{51-54}Met^{55}$	17	12	1.4	0.54	0.017	32	0.9	1.1
Ala^{55}	21	19	1.1	0.57	0.020	28	3.1	2.7

[a] Ligand binding was measured at pH 7, 20 °C, whereas autooxidation and hemin dissociation were measured at pH 5, 37 °C.

one β subunit cistron expressed from a single operon under control of the pTac promotor in the pKK223-3 expression vector (Hoffman et al., 1990). The α and β genes were mutated by replacing the N-terminal valines with methionines, which results in one major translation product (Komiyama et al., 1991). The hemoglobin D-helix double mutant, $\alpha(+D)\beta(-D)$, was constructed at Rice University from the original D-helix mutant genes described in Komiyama et al. (1991).

JM109 *E. coli* cells harboring the wild-type or mutant hemoglobin vectors were grown to an OD_{600} of 2.0−3.0 using "terrific broth" and 0.017 M KH_2PO_4/0.072 M K_2HPO_4 buffer in a 14 L fermentor [see Looker et al. (1994)]. Hemoglobin expression was induced with IPTG (300 μM), and exogenous heme, predissolved in 0.1 M NaOH, was added to a final concentration of 30 mg/mL. The cells were grown for an additional 4.0 h, harvested by centrifugation, weighed, and then frozen in a −70 °C freezer. The cells were resuspended in CO equilibrated lysis buffer (40 mM Tris base/1.0 mM benzamidine, pH 8.0; 3.0 mL/1.0 g of cell paste). Lysozyme was added to 1.0 mg/1.0 g of cell paste and incubated with the paste for 20 min at 10 °C. After blending, Mg^{2+} and Mn^{2+} were added to final concentrations of 0.01 and 0.001 M, respectively. Then DNase was added (20.0 mg/mL of lysate), and the mixture was incubated for 20 min at 10 °C. DNA was precipitated by slow addition of 10% poly(ethylenimine) in distilled water to a final concentration of 0.05%. The lysate was stirred for 15 min and then centrifuged for 20 min at 14000*g*. The supernatant was then concentrated and its conductivity adjusted to that of the buffer used in the first column step. Purification of the recombinant hemoglobins involved three chromatographic steps: (a) a fast-flow Q Sepharose (Sigma) column equilibrated with 20 mM Tris-HCl/0.1 mM TETA, pH 7.4, and eluted with the same buffer; (b) a fast-flow Q Sepharose column equilibrated with 20 mM Tris-HCl, pH 8.5, and eluted with a pH gradient from 8.5 to 7.0 containing 20 mM Tris-HCl; and (c) a fast-flow S Sepharose column equilibrated in 20 mM Na_2HPO_4, pH 6.8, and eluted with a pH gradient from 7.0 to 8.0 containing 20 mM sodium phosphate [see Looker et al. (1994)].

Hemin Dissociation. Time courses for hemin dissociation were measured by reacting 6.0 μM metmyoglobin or methemoglobin with excess H64Y/V68F apomyoglobin as described by Hargrove et al. (1994a). The reactions were measured at 37 °C in 0.15 M buffer/0.45 M sucrose at either pH 5.0 (sodium acetate) or pH 7.0 (potassium phosphate). The myoglobin dissociation time courses were fitted to a one-exponential expression to obtain the hemin dissociation rates, k_{-H}. Hemoglobin dissociation time courses are biphasic and were fitted to a two-exponential expression with equal amplitudes. Hargrove et al. (1994a) have assigned the fast phase to hemin loss from β subunits and the slow phase to hemin loss from α subunits.

Kinetic Measurements. Association and dissociation rate constants for ligand binding to the mutant myoglobins and R-state hemoglobins were determined as described by Rohlfs et al. (1990) and Mathews et al. (1989), respectively. All myoglobin rates were measured in 0.1 M potassium phosphate and 1.0 mM EDTA, pH 7.0 at 20 °C. All hemoglobin rates were measured in 0.1 M Bis-Tris, 0.1 M KCl, and 1.0 mM EDTA, pH 7.0 at 20 °C. The rate constants for the last step in ligand binding to hemoglobin (Hb_4X_3 + XHb_4X_4) are assigned to the R quaternary conformation (Mathews & Olson, 1994).

X-ray Crystallography of Myoglobin D-Helix Mutants. Crystallization of the myoglobin D-helix deletion mutant, Mb(−D), occurs in 2.15 M ammonium sulfate and 20 mM glycine, pH 9.75. The space group is $P2_12_12_1$ with unit cell constants a = 40.26 Å, b = 64.71 Å, c = 50.94 Å, and $\alpha = \beta = \gamma = 90°$. Crystallization of the $Ala^{51-54}Met^{55}$ myoglobin mutant occurs in 2.8 M ammonium sulfate, 1% PEG 2000 monomethyl ether, and 50 mM sodium citrate, pH 6.0. These crystals are in space group $P6_522$ with unit cell constants $a = b$ = 72.8 Å, c = 126.5 Å, $\alpha = \beta = 90°$, and $\gamma = 120°$.

Diffraction data were collected from both crystal forms on a Rigaku R-AXIS II imaging plate detector, with X-rays being supplied by a Siemens rotating anode X-ray generator. Crystals of both the Mb(−D) and the $Mb(Ala^{51-54}Met^{55})$ mutant diffract to a resolution of 2.0 Å. Both structures were solved by molecular replacement and later refined using the X-PLOR software package (Brünger, 1987).

RESULTS AND DISCUSSION

Properties of D-Helix Mutants of Myoglobin. Deletion of the D-helix from sperm whale myoglobin has only a small effect on ligand binding. As shown in Table 1, Mb(−D) has K_{O_2} and K_{CO} values which are only 2-fold less than those for wild-type myoglobin. In contrast, a dramatic 70-fold increase in the rate of hemin dissociation is observed when the D-helix is deleted (Figure 2A). The Mb(−D) mutation also increases the rate of MbO_2 autooxidation 5-fold at low pH. The properties of $Mb(Ala^{51-55})$, $Mb(Ala^{51-54}Met^{55})$, and $Mb(Ala^{55})$ were examined to determine whether the loss of stability due to the D-helix deletion is a result of the change in spacing between the CD corner and the E-helix or is sequence specific (Table 1, Figure 2A). The Ala^{51-55} mutation partially restores resistance to hemin loss and autooxidation, but the rate constant for hemin dissociation is still 20-fold greater than that for wild-type myoglobin. In contrast, the $Ala^{51-54}Met^{55}$ mutation completely restores wild type functionality and stability, implying a key role for the methionine residue at the *D5* position. The single Met^{55} to Ala mutation causes only 5-fold and 3-fold increases in the rates of hemin loss and autooxidation, respectively. Thus, the remainder of the D-helix is also important for stabilizing bound heme, presumably by forming the appropriately sized

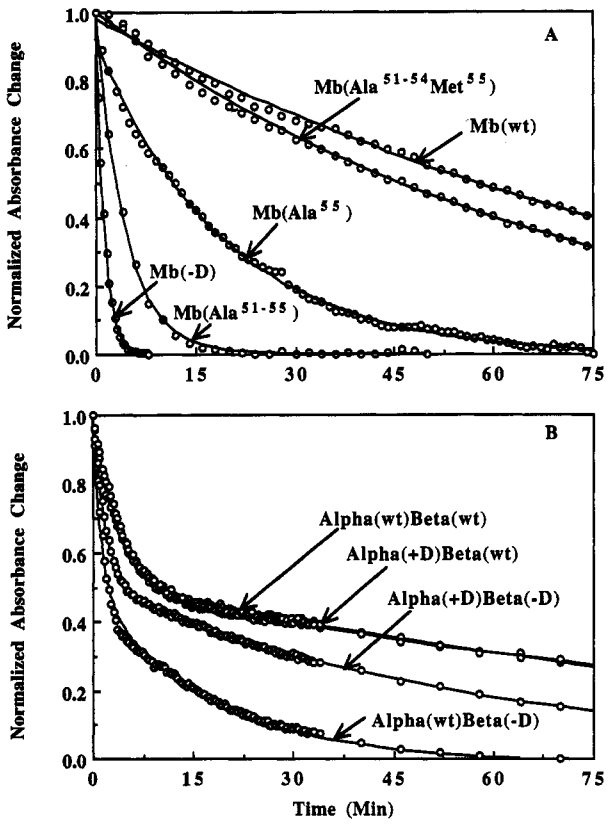

FIGURE 2: (A) Time courses for hemin dissociation from D-helix mutants of sperm whale myoglobin. The symbols represent real data and the lines fits to a single-exponential expression. −D refers to myoglobin missing residues 51−55; wt, to wild type. Absorbance changes were measured at 410 nm. The hemin concentrations for the hemoglobin and myoglobin mutants were 6 μM, and the concentration of the H64Y/V68F apomyoglobin reagent was 24 μM. (B) Time courses for hemin dissociation from D-helix mutants of hemoglobin measured in 0.15 M sodium acetate and 0.45 M sucrose at pH 7.0 and 37 °C. The symbols represent real data and the lines fits to two-exponential expressions.

loop region between the C- and E-helices (Figure 1).

Crystal Structures of the D-Helix Deletion and Ala$_4$Met Replacement Mutants of Sperm Whale Myoglobin. The Mb(−D) and Mb(Ala$^{51-54}$Met^{55}) mutants crystallize in space groups $P2_12_12_1$ and $P6_522$, respectively. Ribbon drawings of structures of these mutants and wild-type aquometmyoglobin are shown in Figure 3.[2] Two key features are apparent when the wild-type and mutant myoglobins are compared. First, the structures on the proximal side of the heme group are virtually identically for all three proteins. The positions of the distal residues surrounding the coordinated water molecule, Leu^{29}(B10), Phe^{43}(CD1), His^{64}(E7), and Val^{68}(E11), are also similar. This conservation of side-chain position in the "first shell" of amino acids near the iron atom explains why deletion of the D-helix and its replacement with alanine residues cause little change in O_2 and CO binding (Table 1).

Second, the electron density between the B- and E-helices is poorly defined in the Mb(−D) mutant.[2] This indicates either multiple conformations or a high degree of thermal motion. This increased flexibility allows much more rapid hemin dissociation, increases accessibility of the distal pocket to solvent molecules, and enhances autooxidation. Similar increases in the rate of hemin dissociation and autooxidation occur when Phe46(CD4) is replaced with smaller amino acids, creating a "hole" in this region of the CD corner (Lai et al., 1995).[3] In contrast, the tertiary structure of the Mb(Ala$^{51-54}$Met^{55}) mutant is well defined and very similar to the wild-type protein. As shown in Figure 3, the conformations of residues Phe^{43}(CD1), Phe^{46}(CD4), Met^{55}(D5), and His^{64}(E7) in the Mb(Ala$^{51-54}$Met^{55}) mutant are virtually identical to those in the wild-type protein, which accounts for the similar rates of ligand binding, hemin dissociation, and autooxidation of the two proteins. Thus, Met^{55} appears to be critical for the formation of a D-helix and the stability of the CD corner, both of which are important in conferring resistance to hemin loss.

Properties of the D-Helix Mutants of Hemoglobin. The dramatic hemin loss results observed for myoglobin led us to reinvestigate the ligand binding and stability properties of the D-helix mutants of human hemoglobin that were made by Komiyama et al. (1991). As shown in Table 2, removal of the D-helix from β subunits or addition of this five-residue sequence to α subunits has only a moderate effect on O_2 and CO binding in R-state hemoglobin. The biggest change is observed for the double mutant, α(+D)β(−D), for which the α and β K_{O_2} and K_{CO} values are 2- and 4-fold less, respectively, than the corresponding constants for wild-type hemoglobin. Komiyama et al. (1991) reported previously that these substitutions have little effect on the equilibrium curve for O_2 binding to recombinant hemoglobin. This observation led them to propose that removal of the D-helix is a relatively neutral mutation with respect to ligand binding, and our kinetic results support this conclusion.

Time courses for hemin dissociation from wild-type and mutant aquomethemoglobin under physiological conditions are shown in Figure 2B. Bunn and Jandl (1968) and later Hargrove et al. (1994a) have shown convincingly that the rapid phase represents hemin loss from β subunits and the slow phase, hemin loss from α subunits. Removal of the D-helix from β subunits has a dramatic effect. In the α-(wild type)β(−D) hybrid, the rate of hemin loss from β subunits increases greater than 3-fold, and the rate from α subunits increases 8-fold (lower curve, Figure 2B, and Table 3). The resulting α(wild type)β(−D) apoglobin is also very unstable and tends to precipitate during the hemin loss assay, making precise measurement of the slow phase difficult. The simplest interpretation of these results is that removal of the D-helix increases the rate of hemin loss and causes unfolding of the β subunit. The remaining holo α subunits in this partially denatured semiglobin are destabilized due to loss of interactions with native β globin partners and lose hemin rapidly. This interpretation is supported by the observation that isolated α subunits lose hemin much more rapidly than when they are coupled to β subunits in hemoglobin (~30−60 h^{-1} *versus* 0.3−0.5 h^{-1}, respectively, at pH 7, 37 °C.[4] In addition, the expression yield of α(wild type)β(−D) hemoglobin is much lower than that of wild type and any of the

[2] A more detailed description of the crystal structures of the Mb(−D) and Mb(Ala$^{51-54}$Met^{55}) mutants is being prepared for publication by M. B. Berry, E. I. Ho, T. L. Whitaker, J. S. Olson, and G. N. Phillips, Jr.

[3] The F46A and F46V sperm whale myoglobin mutants show rate constants for hemin dissociation equal to 13 and 23 h^{-1}, respectively, at pH 5.0, 37 °C (M. Hargrove, unpublished observations). These values are ~10−20-fold greater than the corresponding rate constant for wild-type myoglobin (Table 1).

[4] M. S. Hargrove, A. J. Mathews, and J. S. Olson, manuscript in preparation on the effects of subunit aggregation on the rate of hemin loss from the α and β subunits of human hemoglobin.

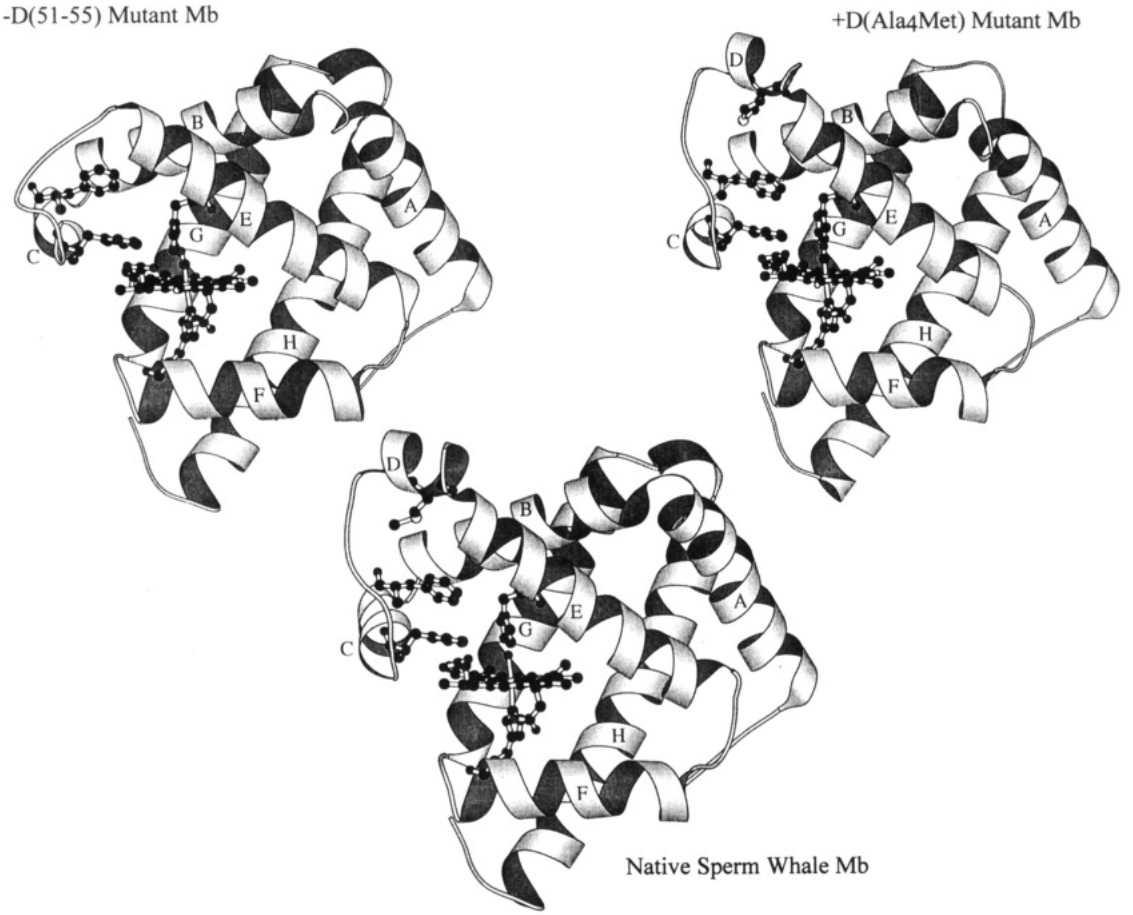

FIGURE 3: Ribbon drawings based on the three-dimensional crystal structures of wild-type sperm whale myoglobin, Mb(−D), and Mb(Ala$^{51-54}$Met^{55}). Note that the distal pocket residues are in the same positions for all three proteins but that the conformation of the CD corner is quite different in the Mb(−D) mutant.

Table 2: R-State Hemoglobin α Subunit and Hemoglobin β Subunit Rate and Equilibrium Constants for Oxygen and Carbon Monoxide Binding at 20 °C in 0.1 M Bis-Tris, 0.1 M KCl, and 1.0 mM EDTA, pH 7.0

hemoglobin	k'_{O_2} (μM^{-1} s^{-1})	k_{O_2} (s^{-1})	K_{O_2} (μM^{-1})	k'_{CO} (μM^{-1} s^{-1})	k_{CO} (s^{-1})	K_{CO} (μM^{-1})
			Hemoglobin α Subunit			
α(native)β(native)	23	11	2.0	2.7	0.0089	300
α(wt)β(wt)	19	15	1.3	2.2	0.0086	260
α(+D)β(wt)	24	21	1.1	1.7	0.0230	74
α(wt)β(−D)	21	14	1.5	2.3	0.0081	280
α(+D)β(−D)	13	13	1.0	1.7	0.014	120
			Hemoglobin β Subunit			
α(native)β(native)	79	28	2.8	7.6	0.011	690
α(wt)β(wt)	74	47	1.6	5.9	0.010	590
α(+D)β(wt)	75	43	1.7	5.1	0.013	390
α(wt)β(−D)	110	58	1.8	7.5	0.017	440
α(+D)β(−D)	25	41	0.61	5.0	0.027	180

Table 3: Rate Constants for Hemin Loss and Autooxidation of D-Helix Hemoglobin Mutants at 37 °C and 3−6 μM Heme[a]

Hb mutant	α k_{-H} (h^{-1})	β k_{-H} (h^{-1})	k_{ox} (h^{-1})
α(wt)β(wt)	0.42	16	0.90
α(+D)β(wt)	0.44	15	0.84
α(wt)β(−D)	3.3	48	5.4
α(+D)β(−D)	1.0	33	3.0

[a] Hemin dissociation was measured at pH 7, whereas oxidation was measured at pH 5.

other D-helix hemoglobin mutants, indicating an unstable apoglobin and/or poor heme binding (Hargrove, 1994b).

Insertion of the D-helix into α globin had no effect on the rates of hemin loss when wild-type β subunits were present in the hemoglobin hybrid. However, the α(+D)β(−D) double mutant is significantly more resistant to hemin loss and precipitation than α(wild type)β(−D) (middle curve, Figure 2B, and Table 3). In addition, the expression yield of α(+D)β(wild type) hemoglobin was approximately 3-fold greater than α(wild type)β(wild type), both of which were much greater than that of the unstable α(wild type)β(−D) mutant. Thus, although addition of an α D-helix does not decrease the rate of hemin loss from hemoglobin, it may still stabilize the α apoglobin structure. This would explain the enhanced expression yields of the α(+D)β(wild type) hybrid and low rates of hemin loss from the α(+D)β(−D) hybrid compared to the α(wild type)β(−D) mutant.

Overall rates of autooxidation of the recombinant hemoglobins are also listed in Table 3. These reactions were carried out at pH 5 in order to speed up the reaction and allow more accurate comparisons. At pH 7 hemoglobin

autooxidation occurs over several days even at 37 °C (Zhang et al., 1991). The time courses at pH 5.0 appear to show two phases with rates that differ by a factor of 2−5. This may be due to differences between the α and β subunits at low pH, but the results for the D-helix hybrids are not readily interpreted in terms of chain differences. Both subunits in the α(wild type)β(−D) and α(+D)β(−D) hybrids autoxidize more rapidly than those in the wild-type control and the α-(+D)β(wild type) mutant. Consequently, only the rates determined by fitting to a single exponential are presented in Table 2. These data show that removing the D-helix from β subunits enhances hemoglobin autooxidation ~5-fold at low pH.

Conclusions. Under physiological conditions, denaturation of hemoglobin and myoglobin occurs in three steps: (1) autooxidation to the aquomet form in which the Fe^{3+}-His^{93}-(F8) bond is much weaker than in the ferrous state; (2) hemin dissociation, which is usually irreversible since the prosthetic group is not very soluble at neutral pH; and (3) unfolding which often leads to precipitation and degradation of the apoglobin. The reverse of these processes must occur following translation of hemoglobin and myoglobin genes *in vivo*. Hargrove et al. (1994b) have shown that both globin stability and resistance to hemin loss are key determinants of holomyoglobin expression yields in *E. coli*. Thus, there is strong selective pressure to preserve structural elements that inhibit autooxidation and hemin dissociation.

The results in Tables 1−3 and Figure 2 show that the D-helix in myoglobin and hemoglobin β subunits is required for inhibition of hemin loss and autooxidation, even though it plays little role in regulating oxygen binding. Addition of a D-helix to α subunits causes no change in the rate of hemin loss from hemoglobin with wild-type β subunits. On the other hand, the rate of hemin loss from α subunits increases almost 10-fold when paired with β(−D) subunits, and the presence of an α D-helix does decrease markedly the rate of hemin loss from the α(+D)β(−D) hybrid. When α subunits are completely separated from β subunits, the rate of hemin dissociation increases 100-fold compared to that observed for α subunits in either dimers or tetramers.[4] Taken together, these results suggest that the absence of a D-helix in the α subunit of chordate hemoglobins is compensated by interactions with adjacent β subunits which stabilize the region between the C- and E-helices. These stabilizing interactions appear to be indirect and occur through the $\alpha_1\beta_1$ interface involving the G-, H-, and B-helices of both subunits since the rate of hemin loss from α subunits is little affected by tetramer dissociation into dimers.[4] Thus, in the presence of normal β subunits, there is little selective pressure to preserve the D-helix region of the α subunit gene.

The crystal structure of yellowfin tuna myoglobin reported by Birnbaum et al. (1994) shows that the region between residues 50−55 exists as a random coil and that Met^{55} is replaced by Ile. This is consistent with our observation that Met^{55} in sperm whale myoglobin is an important residue in maintaining the stability of the D-helix and the CD corner loop region (Table 1). The D-helix is absent in both the α and β subunits of hemoglobins from *Squalus acanthias* (dogfish shark) and *Heterodontus portusjacksoni* (Port Jackson shark) (Aschauer et al., 1985). Thus, selective pressure to maintain the D-helix may become less significant if other portions of the globin molecule or adjacent subunit partners provide enough stability for expression and function.

Several natural hemoglobin variants have been reported with internal deletions in their primary sequences, indicating that this type of mutation is not a rare evolutionary event (Bunn & Forget, 1987). Despite their locations in loop regions, these deletions result in unstable proteins and are unlikely to become fixed in a large population without additional changes. Our experiments show that the conformation of the CD loop region and the rate of hemin loss can be affected greatly by a single amino acid residue, Met^{55}. This suggests that deletions and insertions in loop regions are compensated by secondary point mutations which maintain or enhance the stability of the original protein molecule. Since loop regions often occur in ligand binding sites, their ability to accept deletions, insertions, and point mutations is critical for the evolution of diversity in protein function.

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BI950837K