Aliphatic Semisynthetic Variants of the Amino-Terminal Residue of Sperm Whale Myoglobin: Enrichment with ^{13}C and Determination and Interpretation of Terminal pK Values†

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Received April 8, 1985

ABSTRACT: The synthesis of a series of myoglobins substituted in the amino-terminal residue to provide variation in the aliphatic nature of the side chain and enrichment in ^{13}C was accomplished by semisynthetic methods. The replacements for valine, the native first residue, included ^{13}C-enriched glycine, alanine, valine, leucine, and isoleucine. The products were extensively characterized and found to be virtually indistinguishable by most physical methods. ^{13}C NMR spectroscopy showed significant differences in the amino-terminal pK value, ranging from 7.72 for [Gly^1]myoglobin to 7.15 for [Leu^1]myoglobin. Consideration of the electrostatic effects of the charge matrix indicated a balance of interactions at this site not significantly altered by these variations in the side chain. By examination of the crystal structure, consideration of earlier work regarding the interactions of the side chain of Leu-2, and data regarding the motions of the terminal residue, it was concluded that the interaction of the side chain of the first residue with the hydrophobic cluster formed primarily by close contact of invariant residues Leu-2 and Leu-137 was the primary cause for the reduction in terminal pK values seen for the larger aliphatics. By restricting the freedom of the residue, this interaction limits the available hydration volume and consequently favors the unprotonated form of the amine. The concurrent observation of both functional elements in the series of α-amino-terminal residues brings out the interrelated consequences for the two categories of solvent interactions controlling structural and functional properties in a graded way.

Assessing the role of a specific amino acid at a given site in defining the structural and functional properties of a protein is a fundamental goal of protein chemistry. While natural variants have provided evidence useful to this end, these variants are necessarily limited to changes that are not critically maladaptive. Protein semisynthesis offers the opportunity to introduce variations at a chosen site and to design these variations to allow a systematic examination of the properties influenced by the nature of that residue. Covalent reconstruction of the protein by chemical or enzymatic means, utilizing a substantial portion of the original structure as an intermediate, allows incorporation of these variations with minimal risk of error. Differences exhibited by the semisynthetic proteins in structure, function, or dynamics, integrating the properties of the residues in the sequence, can be unambiguously attributed to the experimentally introduced variants.

A substantial number of myoglobins from mammalian species have been sequenced. Many of the residues forming the heme pocket have been shown to be invariant or nearly so (Lehman, 1980), although invariant residues can be found throughout the molecule. Leucine in the second position is invariant, and glycine predominates at the amino terminus, with valine being the only alternative and itself limited to certain cetacean species (Bogardt, 1978; Bogardt et al., 1980). The highly conserved nature of these residues may suggest some specific structural significance.

Development of a chemical procedure for the selective removal of the amino-terminal residue of sperm whale myoglobin, which minimizes the use of organic solvents and consequential irreversible denaturation of the protein (DiMarchi et al., 1980), has led to the present study. This paper details the synthesis of five myoglobins incorporating an aliphatic series of ^{13}C-enriched amino acids at the amino terminus. The amino acids were chosen to introduce stepwise variation in the aliphatic character of the amino-terminal side chain. Uniform ^{13}C enrichment of the amino acid was used in all cases with the exception of the [Gly^1]myoglobin, where the α-carbon was enriched to 90%. The proteins were extensively characterized to verify the fidelity of the semisynthetic protocol, with further studies to assess the consequences of the imposed variations.

Isotopic enrichment made possible both the analysis of motional behavior of the terminal residue and the measurement of the pK of its α-amino group. Relative restriction of movement compared with the glycine form was associated with the alanine and, particularly, the valine, isoleucine, and leucine forms. In these last preparations, the α-amino pK values were substantially lower than those for the alanine and, especially, the glycine forms. The basis of the correlation between motional behavior and pK may lie in the selective limitation of the hydration shell of the amino group in the more closely tethered hydrophobic amino acids. Such limitation of the hydration shell will favor the deprotonated amine.

Experimental Procedures

The principal component of sperm whale myoglobin was isolated and purified as previously described by Hapner et al. (1968) and was used as the starting material for all semisynthetic preparations. The myoglobin was maintained in the

† This is Paper 134 in a series dealing with coordination complexes and catalytic properties of proteins and related substances. This work was supported by U.S. Public Health Service Research Grants HL-05556 and HL-14680.

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0006-2960/85/0424-6707$01.50/0

ferri form unless otherwise noted. Experimental methods for amino acid analysis, cellulose acetate electrophoresis, potentiometric hydrogen ion titrations, sequence determinations, and ultraviolet–visible spectroscopy are described elsewhere (DiMarchi et al., 1978, 1979). Purifications of products and intermediates by cation-exchange chromatography were typically performed with CM-Sephadex C-50 resin in phosphate buffer, ionic strength 0.01, pH 6.5, with anion-exchange chromatography performed with DEAE-Sephadex A-50 in 0.1 M Tris-HCl,[1] pH 9.2. Acid-stability measurements were performed according to the procedures of Flanagan et al. (1983) at 0.01 M ionic strength. ^{13}C-enriched glycine (90% ^{13}C at the α-carbon) was obtained from Merck. Uniformly enriched L-amino acids (20% ^{13}C), alanine, isoleucine, and valine, were obtained from the Los Alamos National Laboratory. Leucine and additional valine (15% U-^{13}C) were purchased from Cambridge Isotope Laboratory. Silica gel plates (5 × 10 cm) were used for thin-layer chromatography of amino acid derivatives.

Preparation of Des-Val^1*-acetimidomyoglobin Intermediates.* The reaction of myoglobin with methyl acetimidate followed DiMarchi et al. (1978), utilizing conditions favoring complete reaction of lysines but maintaining a free amino terminus. The extent of lysine protection was determined by reaction of an aliquot of the protein with 3,4,5,6-tetrahydrophthalic anhydride (THP anhydride) and subsequent analysis by cellulose acetate electrophoresis at pH 9.2. The acetimidation reaction was repeated if the analysis showed less than 55% of the protein bearing complete lysine protection. This derivative of the protein can be substantially purified by anion-exchange chromatography (DiMarchi et al., 1978) and was used for the subsequent amino-terminal cleavage protocol in the synthesis of [Ala^1]myoglobin. In all other cases, the acetimidation reaction products underwent the terminal cleavage reaction steps prior to purification of the intermediate or isolation of the protein with complete lysine protection. Removal of the amino-terminal valine follows DiMarchi et al. (1980) with minor modifications. Following conversion to the (carbonmonoxy)ferro form, 2-g aliquots of the protein were treated under nitrogen with 3-sulfophenyl isothiocyanate (3-SPITC), an Edman reagent with enhanced solubility in aqueous conditions (Dwulet & Gurd, 1976). The reaction mixture was subsequently deionized, oxidized with potassium ferricyanide, and deionized once more. The protein solution was made 10 mM in sodium fluoride and acidified to pH 3.5 for extraction of the heme with ice-cold butanone. The apoprotein was then lyophilized, either directly after extraction of the heme or following a short dialysis to remove residual butanone.

The terminal valine was cleaved as described (DiMarchi et al., 1979) by using 200 mg of dithioerythritol, 100 μL of anisole, and 10 g of trifluoroacetic acid (TFA) per gram of protein. The TFA was removed by rotary evaporation followed by vacuum. The protein was then rehydrated and dialyzed extensively.

The optimal amount of heme for reincorporation was determined by mixing aliquots of the apoprotein with hemin chloride in molar ratios varying from 0.75 to 1.25, followed by dilution with phosphate buffer, and determination of the maximum 409/280-nm absorbance ratio. Hemin chloride was added to the apoprotein solution at pH 10.8 by addition in 0.1 N sodium hydroxide, and the solution was subsequently dialyzed. Incorrectly reconstituted protein was removed by precipitation following dialysis against phosphate buffer. The protein was subsequently purified on CM-Sephadex C-50 resin in pH 6.5 phosphate buffer. The resulting des-Val^1-acetimidomyoglobin was used in the preparation of [Gly^1]myoglobin and [Ala^1]myoglobin.

The isolation of des-Val^1-N^{ϵ}_{19}-acetimidomyoglobin followed the procedure for reaction with THP anhydride (DiMarchi et al., 1978) and subsequent purification of the des-Val^1-N^{α}-THP-N^{ϵ}_{19}-At-myoglobin, exploiting the charge change introduced by the reaction of the anhydride with the free amino groups as a chromatographic handle. Removal of the THP group at the amino terminus by dialysis against citrate buffer at pH 5.5 was followed by purification by cation-exchange chromatography. The product, des-Val^1-N^{ϵ}_{19}-At-myoglobin was isolated in yields averaging 23% and was used in the synthesis of myoglobins with valine, leucine, and isoleucine at the amino terminus.

Synthesis of [[2-^{13}C*]-Gly*^{1}*]myoglobin (Neireiter, 1979).* TFA-Glycine was prepared by reaction of [2-^{13}C]glycine with trifluoroacetic acid anhydride in trifluoroacetic acid (Neireiter, 1979; DiMarchi et al., 1978) and isolated as crystals in 75% yield, mp 114–116 °C. Subsequent activation of the carboxyl group by dicyclohexylcarbodiimide- (DCC-) mediated formation of the *N*-hydroxysuccinimide (ONSu) ester (Anderson et al., 1964) gave the active ester in 42% yield, mp 135.5–137 °C. The des-Val^1-At-myoglobin at 2.5% concentration underwent reaction with a 10-fold excess of TFA-Gly-ONSu at pH 7.5, 15 °C for 2 h. After dialysis to remove excess TFA-Gly, the protecting groups were simultaneously removed by addition of the protein to a mixture of concentrated ammonium hydroxide–acetic acid (30:1 v/v) at 25 °C, pH 11.8 for 13 h. Following dialysis, the protein was purified on CM-Sephadex C-50 in pH 6.5 phosphate buffer. Further purification was performed with DEAE-Sephadex A-50 at pH 9.1 in Tris-HCl buffer. Yield of the product from the coupling step was 29%.

Synthesis of [[U-^{13}C*]Ala*^{1}*]myoglobin.* [[[(Methylsulfonyl)ethyl]oxy]carbonyl]alanine (MSC-Ala) was prepared following the procedures of Tesser & Balvert-Geers (1975). Analysis of the reaction by thin-layer chromatography (TLC) in chloroform–methanol–acetic acid–water (60:30:1:4) showed a single spot (R_f values: Msc-Ala, 0.57; Ala, 0.06). The carboxyl group was activated with the formation of the ONSu ester (Anderson et al., 1964), and the Msc-Ala-ONSu was collected as crystals from 2-propanol with mp of 149.5–152.5 °C. A 5% solution of des-Val^1-At-myoglobin was treated with a 10-fold excess of Msc-Ala-ONSu at pH 7.5, 15 °C for 2 h. The myoglobin was separated from excess Msc-Ala by gel filtration and subsequently purified by ion-exchange chromatography with CM-Sephadex C-50 at pH 6.5. Removal of the protecting groups was accomplished as in the case of [Gly^1]myoglobin, followed by a similar purification scheme, yielding 41% product from the coupling stage.

Synthesis of [[U-^{13}C*]Ile*^{1}*]myoglobin.* Msc-Ile was prepared by reaction of Msc-ONp with isoleucine following the procedures of Tesser & Balvert-Geers (1975). Extent of the reaction was determined by thin-layer chromatography in butanol–acetic acid–water–pyridine (30:6:4:1), with R_f values determined for Msc-Ile and Ile as 0.66 and 0.50, respectively.

[1] Abbreviations: At, acetimidyl; des-Val^1, lacking the valine amino terminus; N^{ϵ}_{19}, modification at the ϵ-amino group of 19 lysines; N^{α}, modification at the α-amino group; THP, 3,4,5,6-tetrahydrophthalyl; Msc, [[(methylsulfonyl)ethyl]oxy]carbonyl; ONSu, *N*-hydroxysuccinimide; TFA, trifluoroacetic acid; T_1, spin–lattice relaxation time; NOE, nuclear Overhauser enhancement; DCC, dicyclohexylcarbodiimide; TLC, thin-layer chromatography; 3-SPITC, 3-sulfophenyl isothiocyanate; 3-SPTC, [(3-sulfophenyl)thio]carbamoyl; SA, static solvent accessibility; $pK_{1/2}$, value at half-titration according to electrostatic calculations; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

Formation of the ONSu active ester followed Anderson et al. (1964). TLC in 9:1 chloroform–methanol (v/v) showed nearly quantitative formation of the active ester (R_f 0.58; R_f of Msc-Ile 0.51).

A 3% solution of des-val^1 N^{ϵ}_{19}-At-myoglobin at 15 °C was slowly adjusted to contain 12% methanol by dropwise addition of methanol with rapid stirring. The pH was adjusted to 7.6, and a 10-fold excess of Msc-Ile-ONSu dissolved in sufficient methanol to raise the final concentration to 15% was added dropwise to the reaction vessel. The pH of the reaction was maintained at 7.5 ± 0.1 for 4.25 h. Excess Msc-Ile was collected by gel filtration of the reaction mixture, and the coupled protein was isolated by cation-exchange chromatography at pH 6.4. Simultaneous removal of Msc and acetimidyl protecting groups was accomplished by exposure of the protein to concentrated ammonium hydroxide–acetic acid (30:1 v/v) at pH 11.5 for 24 h at 25 °C. The product was purified by cation-exchange chromatography, followed by anion-exchange chromatography where necessary. The yield of [Ile^1]myoglobin from the coupling step was 29%.

Synthesis of [[*U*-^{13}*C*]-*Val*^{1}]*myoglobin.* Msc-Val was synthesized according to Tesser & Balvert-Geers (1975), and the extent of reaction was determined by TLC in chloroform–methanol–water–acetic acid (60:30:1:4) (R_f values: Val, 0.36; Msc-Val 0.86). The ONSu active ester was formed following Anderson et al. (1964), with TLC in chloroform–methanol (9:1 v/v) showing nearly quantitative activation (R_f values: Msc-val, 0.45; Msc-Val-ONSu, 0.58).

A 4% solution at 15 °C of des-Val^1-N^{ϵ}_{19}-At-myoglobin was made 12% in methanol by slow addition with rapid stirring. With the pH adjusted to 7.8, a 10-fold excess of Msc-Val-ONSu was added in sufficient methanol to bring the final methanol concentration to 15%. The pH was maintained at 7.5 ± 0.1 for 4.7 h. Excess Msc-Val was removed from the protein by gel filtration, and the protein was purified by cation-exchange chromatography. Deprotection and purification followed the procedures described for [Ile^1]myoglobin, and the resulting yield was 28% from the coupling step.

Synthesis of [[*U*-^{13}*C*]*Leu*^{1}]*myoglobin.* Msc-Leu was synthesized following the procedures of Tesser & Balvert-Geers (1975) and analyzed by TLC in butanol–acetic acid–methanol–pyridine (30:6:4:1) to determine the extent of the reactions (R_f values: Leu, 0.51; Msc-Leu, 0.68). ONSu active ester formation (Anderson et al., 1964) was analyzed in 9:1 chloroform–methanol, and showed nearly quantitative reaction (R_f values: Msc-Leu, 0.54; Msc-Leu-ONSu, 0.75).

Des-Val^1-N^{ϵ}_{19}-At-myoglobin in 4% solution at 15 °C was made 12% in methanol by slow dropwise addition. The pH was adjusted to 7.8, and the active ester in 10-fold excess was added dropwise in methanol to increase the final concentration of the latter to 15%. The pH was maintained at 7.5 ± 0.1 for 2.5 h. Excess Msc-Leu was removed by gel filtration, and the coupled protein isolated by cation exchange chromatography. Deprotection followed as in the previous cases of valine and isoleucine. Purification by cation-exchange chromatography gave the final product in 33% yield from the coupling step.

^{13}C NMR Spectroscopy. ^{13}C NMR spectra were collected with a Bruker WH270 superconducting solenoid operating at 67.9 MHz with a 10- or 15-mm sample tube. Broad-band proton decoupling was used, centered typically at 2.5 ppm (proton) and operating at 3.4–3.7-W total power. Temperature of the sample was controlled at 30 ± 2 °C. Chemical shifts were measured digitally with the instrumental computer relative to internal dioxane at 67.86 ppm, with typical digital resolution of 0.03 ppm. Samples were deionized prior to pH adjustment with saturated phosphate solutions and titrations with 1 N NaOH. The pH values were measured with a Radiometer pHM4c or Corning 135 pH meter equipped with a combination electrode and calibrated with phosphate, borate, and phthalate standard buffers (Bates, 1973). The accuracy of the pH measurements is estimated to be within ±0.005 pH unit. pK values were determined by a nonlinear least-squares fit to a Henderson–Hasselbalch equation.

Results and Discussion

Semisynthetic Procedures. Development of a nondestructive synthetic procedure for the production of des-Val^1-At-myoglobin in good yield (DiMarchi et al., 1980) has allowed the replacement of the amino-terminal valine with a series of amino acids enriched with ^{13}C, with stepwise variation of the aliphatic nature of their side chain. The protocol for the semisynthesis of the five proteins is illustrated in Figure 1. The methods shown are an extension of the semisynthetic procedures of DiMarchi et al. (1979, 1980) and rely on the use of acetimidyl protection of the lysine residues to direct the reaction of the water-soluble Edman reagent, 3-SPITC, and the amino acid active ester to the terminal amino group. Chemical derivatization, electrophoresis, and amino acid analysis are reported in the characterization of the semisynthetic intermediates.

Coupling Conditions. The reactivity of the active esters used for the reincorporation of the amino-terminal residues varied widely with the nature of the side chain and called for different strategies regarding the precise nature of the semisynthetic protein intermediate as well as refinements in coupling reaction conditions for each amino acid. The higher reactivity of the CF_3CO-protected glycine active ester and of Msc-Ala-ONSu allowed direct coupling with des-Val^1-At-myoglobin under aqueous conditions, although further purification after the acetimidation reaction increased the percentage of the protein with complete lysine modification suitable for the coupling with alanine active ester. Reaction with unprotected Lys residues was minimized by the pH of the coupling reaction, and the minor byproducts were removed in the subsequent purifications.

The bulkier, hydrophobic nature of the side chain in active esters of isoleucine, valine, and leucine resulted in reduced reactivity and solubility in aqueous conditions, despite the use of Msc protection and ONSu activation, each contributing to an enhancement of aqueous solubility. To compensate for the reduced solubility, methanol was used as a 15% cosolvent in these couplings without significant deleterious effects on the protein (Asakura et al., 1977). Additionally, des-Val^1-N^{ϵ}_{19}-At-myoglobin was isolated for use in these couplings to eliminate any possible side-chain coupling with its consequent reduction in yield of the desired product.

Refinement of reaction conditions for isoleucine showed that limiting methanol to 15% as a cosolvent, as opposed to 30% used in preliminary work, substantially reduced an anomalous byproduct with a blocked amino terminus that became apparent after deprotection. Analysis of reaction products at both pH 6.4 and pH 9.2 allowed precise determination of the extent of amino-terminal coupling by exploiting the loss of amino-terminal charge normally evident at the lower pH that accompanies the coupling of the Msc-protected amino acid. Time-course analysis of the coupling reactions showed essentially complete amino-terminal coupling within approximately 4–5 h, with additional reaction time resulting only in additional byproduct formation. Overall, these refinements of the procedure raised the yield from the des-val^1-At-myoglobin from 4.8% in preliminary work to 29% in the case of isoleucine.

Table I: Amino Acid Composition[a] and Amino-Terminal Sequence Analysis

	native	des-1-At	[Gly^1]	[Ala^1]	[Val^1]	[Ile^1]	[Leu^1]
			Amino Acid Composition				
Asp	8	7.9	8.1	8.0	7.9	8.1	8.0
Thr	5	4.6	5.0	4.8	4.9	4.7	4.9
Ser	6	5.2	5.9	5.8	5.5	6.4	5.8
Glu	19	18.2	19.0	18.9	18.9	18.4	19.6
Pro	4	ND[b]	4.1	ND	ND	ND	ND
Gly	11	11.0	12.1	10.7	11.2	11.4	11.0
Ala	17	18.0	16.9	18.4	17.6	17.2	17.6
Val	8	6.8	7.0	7.3	8.0	7.1	7.0
Met	2	2.0	1.9	1.8	1.7	2.0	1.9
Ile	9	8.4	9.1	9.6	8.9	9.8	9.2
Leu	18	17.7	18.2	18.3	17.9	18.1	19.2
Tyr	3	2.6	3.0	2.8	2.8	2.6	2.6
Phe	6	5.8	5.9	6.1	5.8	5.7	5.8
Lys	19	1.9	17.9	18.0	18.9	18.2	18.5
His	12	12.0	11.9	11.4	12.6	11.3	11.3
Arg	4	3.9	3.9	4.1	3.8	3.8	4.0
Trp	2	ND	ND	ND	ND	ND	ND
At-Lys	0	19.0	1.2	0.0	0.0	0.0	0.0
			Sequencer Data (Mole Fraction)				
residue 1			0.97	1.00	0.95	0.95	1.00
residue 2 (Leu)			0.97	1.00	0.94	0.94	1.00

[a] Protein hydrolysis of replicate samples was performed for 24, 48, and 72 h. Contents of serine, threonine, and At-lysine were determined by extrapolation to 0 h; with valine, isoleucine, and lysine, contents were determined by extrapolation to 100 h. [b] ND, not determined.

Table II: Spectroscopic Properties

ligand state and wavelength ratio	native	[Gly^1]	[Ala^1]	[Val^1]	[Ile^1]	[Leu^1]
aquoferri						
409/280	5.33	5.28	5.47	5.25	5.14	5.21
cyanoferri						
423/280	3.48	3.44	3.54	3.49	3.50	3.56
360/280	0.91	0.92	0.92	0.92	0.91	0.93
541/280	0.34	0.33	0.34	0.33	0.34	0.33
carboxyferro						
542/579	1.15	1.16	1.13	1.16	1.14	1.12
345/579	2.20	2.24	2.21	2.33	2.17	2.22
280 (CN^-)/280 (H_2O)	1.02	1.02	1.03	1.02	1.03	1.00

Relative reactivity was generally poorer with the sterically hindered β-branched valine and isoleucine. Preliminary studies showed that, under identical reaction conditions, the extent of the coupling step with leucine was nearly double that of isoleucine (56% vs. 33%, respectively).

Characterization of Semisynthetic Products. Amino acid compositions and sequence analysis of the semisynthetic products are shown in Table I, confirming loss of the amino-terminal valine and its replacement by the chemically reincorporated amino acid in each case as intended, as well as removal of the protecting groups. The purity and electrophoretic identity of the semisynthetic proteins was demonstrated with cellulose acetate electrophoresis at pH 6.4 and pH 9.2 as shown in Figure 2. The proteins exhibited ultraviolet and visible absorption spectra indistinguishable from the untreated protein in the aquoferri, cyanoferri, and (carbonmonoxy)ferro ligand states, as seen in Table II. Since the Soret band at 409 nm in the aquoferri form is a sensitive measure of the intact structure (Breslow & Gurd, 1962; Adler et al., 1973), these ratios of extinction coefficients offer strong support that the tertiary structure adopted by the semisynthetic products is not distinguishable from the native, despite the occasionally rigorous conditions necessary for their synthesis.

As a further examination of the tertiary structure, the ^{13}C NMR spectra of the products were collected under closely comparable conditions and carefully examined. The spectra, shown in Figure 3, were found to be nearly indistinguishable with the exception of the prominent resonances attributable to the enriched carbons introduced with the replacement of the respective amino-terminal residue. In addition, comparison of the chemical shifts of eight isoleucine C_{δ_1} resonances observed in the natural abundance spectrum of native cyanoferri myoglobin (Wittebort et al., 1978) with those appearing in the spectra of the semisynthetic myoglobins (Table III) shows no significant deviation in the chemical shifts of these carbon resonances. These natural abundance resonances have been assigned to isoleucine residues that appear in helical segments in a variety of locations in the molecule (Gurd et al., 1982). Three isoleucine C_{δ_1} resonances in [Ile^1]myoglobin are lost to the overlap of the enriched C_{δ_1} resonance of the terminal isoleucine. Since the chemical shift of a nucleus is a sensitive function of its microenvironment, the conformation of the native and semisynthetic proteins can be shown to differ little at a number of reference points (Table III). Additionally, other resonances previously recognized and assigned in the spectra of myoglobins are observed in the underlying natural

Table III: Isoleucine δ-Carbon Chemical Shifts[a]

residue[b]	native[b]	[Gly^1]	[Ala^1]	[Val^1]	[Leu^1]	[Ile^1]
28	14.16	14.16	14.12	14.12	14.14	14.15
30	13.17	13.17	13.12	13.16	13.11	13.16
112	12.85	12.85	12.75	12.85	12.74	12.85
111	12.51	12.48	12.50	12.44	12.47	ND[c]
107	11.75	11.72	11.60	11.72	11.62	ND
142	11.40	11.37	11.40	11.35	11.33	ND
101	9.80	9.89	9.80	9.88	9.88	9.83
75	9.20	9.23	9.10	9.19	9.19	9.17

[a] Chemical shifts in ppm from TMS; all proteins in cyanoferri ligand state, pH 7.2. [b] From Gurd et al. (1982). [c] ND, not determined.

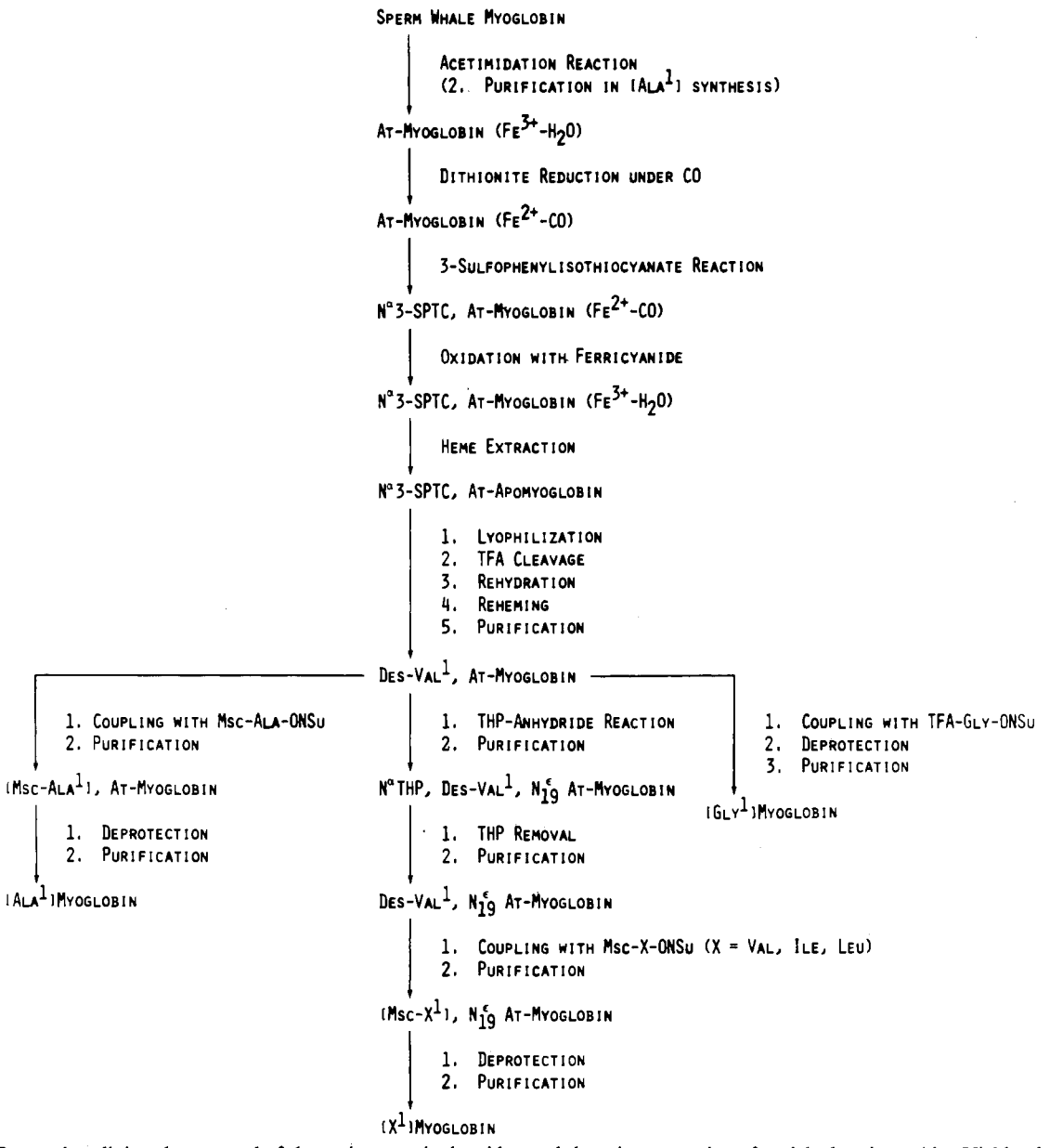

FIGURE 1: Protocol outlining the removal of the amino-terminal residue and the reincorporation of enriched amino acids. Yields of the various intermediates appear in the text, and electrophoretic analysis of intermediates and products is shown in Figure 2.

abundance spectra of the semisynthetic proteins (Wittebort et al., 1978). Examples of these are the resonances of tyrosine and arginine C_ζ carbons at about 158 ppm, tryptophan C_γ resonances near 110 ppm, and numerous peaks of histidine and phenylalanine aromatic carbons between 125 and 140 ppm that appear consistently in the spectra of the semisynthetic proteins.

Acid Stabilities. The range of behavior of the semisynthetic proteins under conditions of acid denaturation at ionic strength 0.01 M is very narrow, with pH_{mid} values of 3.71–3.74, comparable to those seen for the myoglobins of the sperm whales as studied by Flanagan et al. (1983). Although undertaken to examine the possible effects of the substitutions, the acid-stability studies provide further evidence of the success of the semisynthetic protocol, since the common intermediate having the amino terminal removed showed a pH_{mid} value increased by 0.15 pH unit and acetimidation of the 19 lysines increased the value of the pH_{mid} by approximately 0.25 pH unit (DiMarchi et al., 1980). In each case, the substituted amino acid returns the less stable des-Val[1]-At-myoglobin intermediate to a state of conformational stability nearly identical with that of native myoglobin.

Potentiometric Hydrogen Ion Titrations. The potentiometric titrations of the semisynthetic myoglobins at ionic strength 0.01 M are represented in Figure 4. Deviations are centered primarily in the neutral pH region, where [Gly[1]]- and [Ala[1]]myoglobins each exhibit slightly more positive charge than native sperm whale myoglobin in the pH region of the amino-terminal titration. The leucine and isoleucine variants show slightly less positive charge, and the [Val[1]]myoglobin is indistinguishable from the native molecule, all in keeping with the amino-terminal p*K* values determined experimentally for these proteins as presented below.

Amino-Terminal pK Values. The variation in chemical shifts of each enriched resonance with pH was observed for the determination of the p*K* value for the aquoferri form of each semisynthetic protein (Figure 5, Table IV). Standard deviations are typically less than 0.03 pH unit, with exceptions only where the change in chemical shift approaches the 0.03 ppm digital resolution of the spectra. Values are corrected to 25 °C by use of the van't Hoff equation, assuming $\Delta H^\circ = 10.8$ kcal/mol.[2]

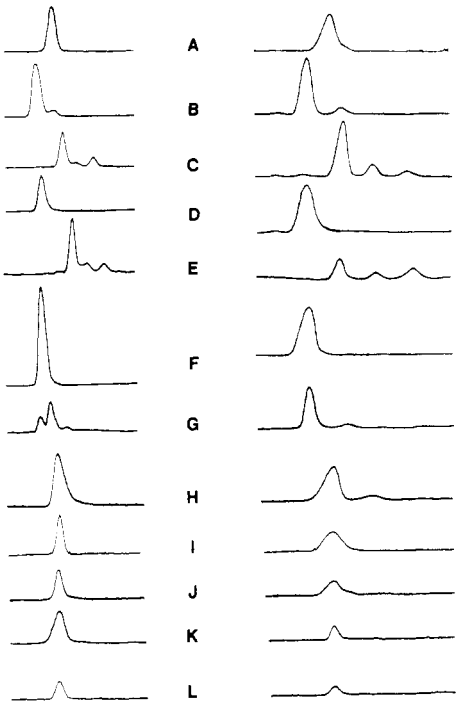

FIGURE 2: Densitometer scans of cellulose acetate electrophoresis of semisynthetic intermediates and products. Results in the left column were obtained at pH 6.4 and those at right at pH 9.2. (A) Sperm whale myoglobin; (B) myoglobin following acetimidation reactions; (C) THP anhydride treated At-myoglobin; (D) purified des-Val^1-At-myoglobin; (E) THP anhydride treated des-Val^1-At-myoglobin; (F) purified des-Val^1-N^{ϵ}_{19}-At-myoglobin; (G) products of coupling reaction of Msc-Leu-ONSu and des-Val^1-N^{ϵ}_{19}-At-myoglobin; (H) [Gly^1]myoglobin; (I) [Ala^1]myoglobin; (J) [Val^1]myoglobin; (K) [Ile^1]myoglobin; (L) [Leu^1]myoglobin. The impurity seen in [Gly^1]myoglobin (H) at pH 9.2 was removed by anion-exchange chromatography prior to further characterization.

Table IV: Amino-Terminal pK Values

	carbon	Δppm	pK (30 °C)	pK (25 °C)
[Gly^1]myoglobin	C_{α}	3.23	7.59 ± 0.01	7.72
[Ala^1]myoglobin	C_O	7.09	7.37 ± 0.02	7.45
	C_{α}	1.19	7.28 ± 0.02	
	C_{β}	3.20	7.32 ± 0.02	
[Val^1]myoglobin	C_O	7.57	7.08 ± 0.02	7.23
	C_{α}	1.76	7.14 ± 0.03	
	C_{β}	1.33	7.07 ± 0.02	
	C_{γ_1}	0.92	7.13 ± 0.03	
	C_{γ_2}	0.48	7.10 ± 0.03	
[Ile^1]myoglobin	C_O	6.93	7.09 ± 0.04	7.22
	C_{α}	1.31	7.15 ± 0.03	
	C_{β}	1.27	7.12 ± 0.03	
	C_{γ_1}	0.253	6.98 ± 0.07	
	C_{γ_2}	0.813	7.12 ± 0.03	
	C_{δ_1}	0.05	6.78 ± 0.25	
[Leu^1]myoglobin	C_O	7.35	7.01 ± 0.03	7.15
	C_{α}	1.38	7.04 ± 0.03	
	C_{β}	3.64	7.02 ± 0.02	
	C_{γ}	0.627	7.02 ± 0.03	
	C_{δ_1}	0.184	7.08 ± 0.07	
	C_{δ_2}	0.236	6.80 ± 0.07	

Since the ^{13}C-enriched semisynthetic [Val^1]myoglobin shows no evidence of being chemically or structurally distinct from the native myoglobin, the pK value determined here should accurately reflect that of native myoglobin. The pK value of

[2] Correction of pK values to 25 °C allows direct comparisons with pK values from other determinations. The constant correction involved does not affect the interpretation. Note, however, that the value of the enthalpy used for the correction may be affected by the phenomena revealed by this work.

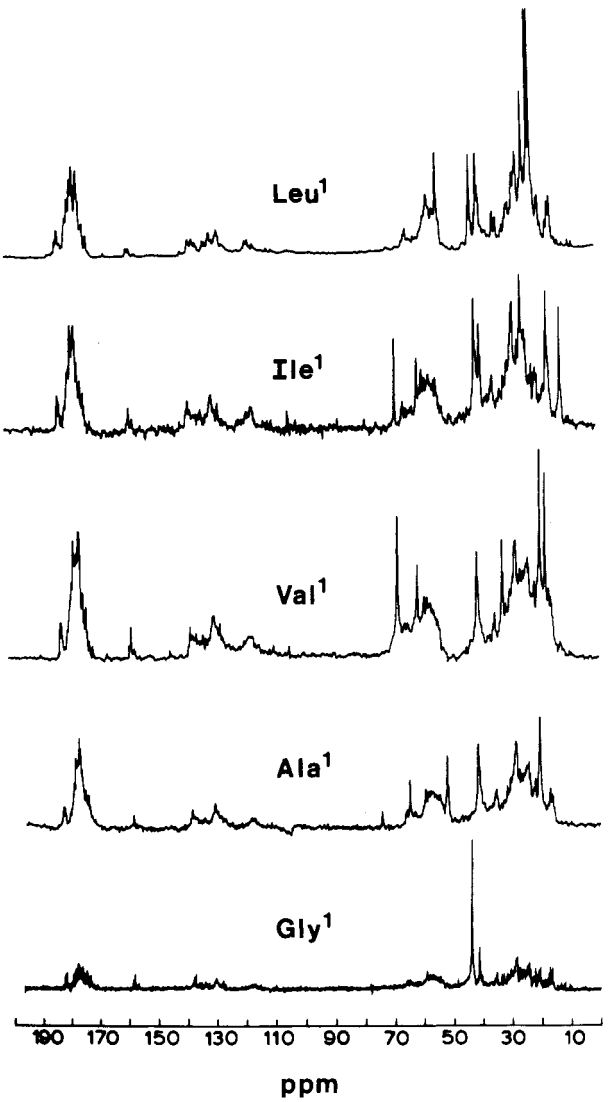

FIGURE 3: Proton-decoupled ^{13}C NMR spectra of the enriched semisynthetic myoglobins. The proteins were in the cyanoferri ligand state, pH 7.2, and the spectra accumulated at 63.5 kG. The ppm scale is referenced to TMS. The prominent resonances at 67.9 ppm in the [Val^1]- and [Ile^1]myoglobins are dioxane used as an internal standard. The carbonyl resonances in the uniformly enriched terminal variants are obscured by the natural abundance spectrum.

7.23 corresponds well with an estmate of 7.2 ± 0.1 by DiMarchi et al. (1979) made by comparison of titration curves of N^{ϵ}_{19}-At-myoglobin with N^{α}-Msc-N^{ϵ}_{19}-At-myoglobin and supersedes values reported in earlier studies using cyanate reaction kinetics.[3]

The pK values of the aliphatic series exhibit a notable change with the changing nature of the terminal side chain. A net change of 0.57 pH unit separates the highest observed value of [Gly^1]myoglobin with its pK of 7.72 from the lowest, [Leu^1]myoglobin with a pK of 7.15. The pK values of [Val^1]- and [Ile^1]myoglobin at 7.23 and 7.22, respectively, approach the value seen for [Leu^1]myoglobin. The pK value for [Ala^1]myoglobin lies intermediate to these extremes at 7.45.

The progressive drop in the pK value of the amino terminus, which results from the stepwise variation of that residue to

[3] The amino-terminal pK value of 7.96 for sperm whale myoglobin as determined by cyanate kinetics (Garner et al., 1973, 1975) may be attributable to the effects of the higher ionic strength of the cyanate reaction conditions on the charge-array characteristics of the protein (B. Garcia-Moreno E., personal communication) as well as the possibility of side reactions with lysine residues.

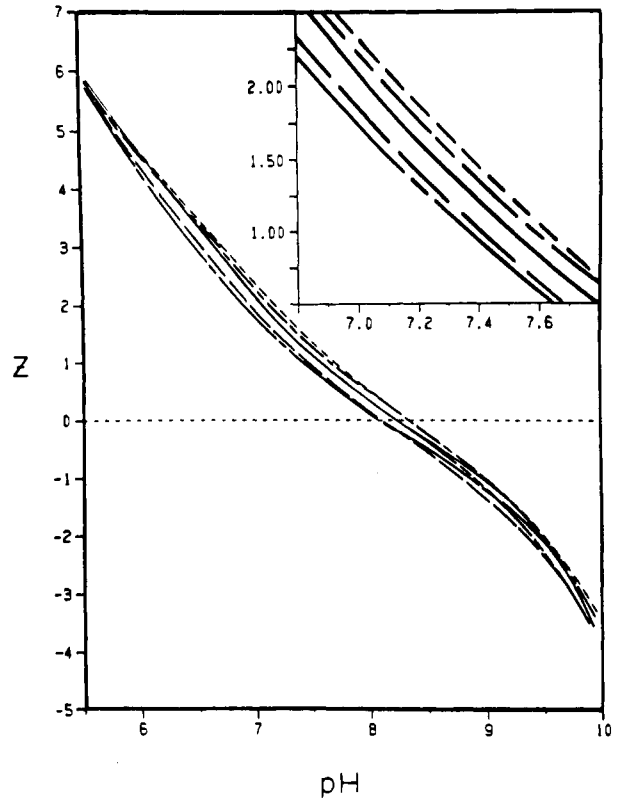

FIGURE 4: Potentiometric hydrogen ion titration curves of the semisynthetic myoglobins: [Gly[1]]myoglobin (---); [Ala[1]]myoglobin (–·–); [Val[1]]myoglobin (– – –); [Ile[1]]myoglobin (– –); [Leu[1]]myoglobin (———); native myoglobin (—). Native and [Val[1]]myoglobin are indistinguishable throughout most of the pH range. From left to right at $Z = 0$, [Leu[1]]myoglobin, [Ile[1]]myoglobin, [Val[1]]myoglobin and native myoglobin, and [Ala[1]]myoglobin and [Gly[1]]myoglobin. The inset shows an enlargement of the region from pH 6.8 to pH 7.8, utilizing the same axes. From left to right in the inset are [Leu[1]]-myoglobin, [Ile[1]]myoglobin, superimposed curves of native and [Val[1]]myoglobin, [Ala[1]]myoglobin, and [Gly[1]]myoglobin.

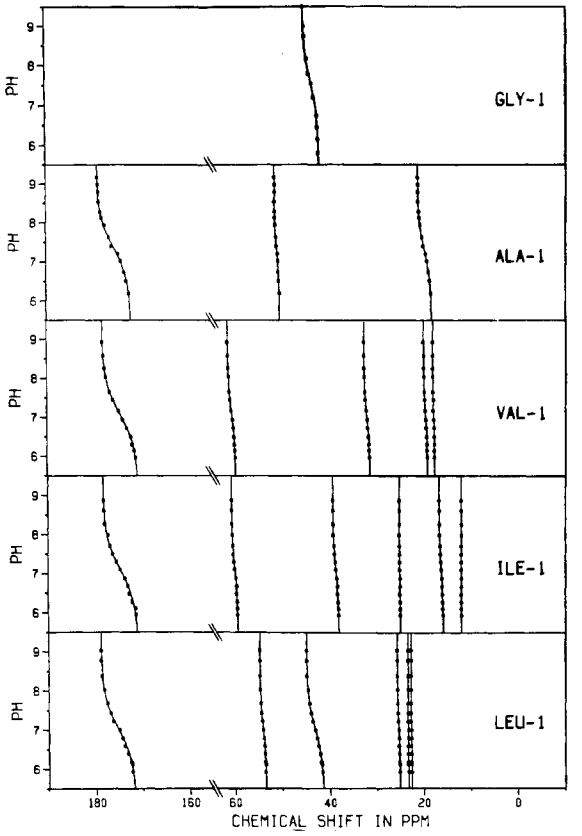

FIGURE 5: Chemical shift variation with pH for the enriched carbon resonances of aquoferri semisynthetic myoglobins. The regression lines shown result from a nonlinear least-squares fit of the data points to the Henderson–Hasselbalch equation, with resulting pK values shown in Table IV.

incorporate largr aliphatic side chains, raises fundamental issues about the interrelations of the traditional categories of stabilizing interactions in proteins and the factors which influence those interactions.

In view of the similarity of all other properties of the series of protein variants with the natural form, including that of stability, and bearing in mind the crucial role of Leu-2 (DiMarchi et al., 1980), it is most unlikely that significant structural differences carry into the first few residues of the sequence. By the same token, the potential interactions of the terminal residue itself can be considered in terms of the native conformation of nearby structures in the molecule contributed by residues in the H helix, for example. Free pentapeptide models representing the terminal sequence with Val or Gly as the amino terminus have been studied and found to have significantly higher α-amino pK values of approximately 7.8 and 8.4, respectively (Garner et al., 1973; Hartzell & Gurd, 1969; Gurd et al., 1971; Neireiter, 1979). The free amino acids, Val and Gly, do not differ significantly in pK values, so that the distinction between the pentapeptides probably reflects the well-known constraints on conformer distributions in peptides with bulky substituents (Morrow et al., 1974; Neireiter, 1979). Note from Table IV, however, that as the segment is brought into the conformational constraints of the native protein structure, the amino-terminal pK value is reduced, the protonated form being relatively destabilized.

Minimal Electrostatic Lattice Interactions of Terminal Residue. The possibility of electrostatic interactions with other charge sites of the protein contributing to the observed effect has been examined by the static accessibility modified Tanford–Kirkwood electrostatic theory (Matthew et al., 1982; Flanagan et al., 1983; Matthew et al., 1985). According to this formalism, the α-amino pK value was found to be essentially unperturbed by its interactions with the charge matrix.[4] The basis for the computed insensitivity of the α-amino pK lies in the balance of all its pairwise electrostatic interactions to produce a net field near zero. A change in SA value for the α-amino group alone would not alter this conclusion, because the charge–lattic interactions would remain balanced (Matthew et al., 1985). As a general example, arbitrarily changing the static solvent accessibility parameter for the α-amino group of the terminal residue from the normal value of 0.90 calculated from the crystal structure to as little as 0.05 resulted in essentially no change of the p$K_{1/2}$ value. In a comparison computation, new coordinates and SA values were generated according to reorientation of the amino-terminal residue by rotation with the C_O–C_α bond. No substantial effect of the bond rotation on the resulting p$K_{1/2}$ was indicated according to the formal treatment. Another aspect of the insensitivity of the interplay of the α-amino-terminal group with the charge–lattice was illustrated by arbitrary variation of the intrinsic pK between 8.0 and 7.5.

Hydration and Hydrophobic Contacts. Because of the balanced net effect of the charge–lattice, the α-amino group can be treated as isolated from the remaining charge sites of the protein. In the following discussion, its titration behavior will be considered to reflect the characteristics of the micro-

[4] The observed pK values thus correspond formally to appropriate intrinsic pK values.

Table V: Correlation of Observed pK Values with Properties of Amino Acids

	amino-terminus and pK of myoglobin					
	Gly, 7.72	Ala, 7.45	Val, 7.23	Ile, 7.22	Leu, 7.15	correlation
pI[a]	5.97	6.00	5.96	6.02	5.98	0.05
pK[a]	9.60	9.69	9.62	9.62	9.60	0.11
ΔG of transfer[b]	−0.34	−0.29	0.09	0.24	−0.12	0.74
scaled vol[c]	0.0	15.9	47.7	63.6	63.6	0.959
scaled polarity[c]	37.0	25.9	8.6	0.0	0.0	0.970
solution enthalpy (kcal/mol)[d]	3.4	1.83	0.9	0.9	1.0	0.989
side-chain length (Å)[e]	0.5	2.5	5.0	5.5	5.5	0.989
side-chain area (Å^2)[f]	0.0	75	133	160	164	0.992
solubility (mol/kg)[d]	3.33	1.86	0.496	0.263	0.165	0.996

[a] Edsall (1943). [b] Wolfenden et al. (1981); based on Chothia (1976). [c] Bogardt et al. (1980). [d] Hutchens (1975). [e] Dawson (1972). [f] Shrake & Rupley (1973).

environment of the residue undergoing titration at the protein surface. The pK value in each protein variant represents the energetics of the protonation–deprotonation-transfer reaction between the terminal α-amino group and bulk solvent, which requires contact through the hydration shell of the group. The phenomena reported here may reflect the influence of the immediate environment on the properties of the hydration solvent.

In order to evaluate which properties of the residue might affect the microenvironment resulting in the observed phenomena, the α-amino pK values of the proteins were listed in correlation with a number of properties of the corresponding free amino acid and examined for linearity of the correlation. The pK and pI values of the free amino acid both fail to show any linearity with the observed pK values, as do a number of other properties of the amino acids as shown in Table V. Those properties showing better correlation include solution enthalpy, length of side chain, surface area of the side chain, and, especially, solubility of the amino acid in water and in general suggest that the interaction with aqueous solvent is central to the phenomenon.

The surface area of the varied side chain can be further examined in terms of hydrophobic free energy, in that each square angstrom of surface area removed from contact with water results in a free-energy gain estimated at about 25 cal/mol (Chothia, 1975, 1976). Examination of side chains on the basis of static solvent accessibility of the crystal structure shows that of 113 Å^2 of surface area in the valine side chain (Shrake & Rupley, 1973), only 60 Å remain accessible to solvent in the protein structure. Contact of the side chain of Val-1 with residues Leu-2 and Leu-137 provides for removal of an additional 24 Å^2 from the solvent-accessible surface area of these aliphatic side chains. These contacts are observed in the crystal structure determined at 80 K, where significantly less flexibility is seen for the amino-terminal region (Hartmann et al., 1982). On this accounting, the magnitude of free-energy gain resulting from these side-chain burials would exceed 2.4 kcal/mol, energetically equivalent to a ΔpK of approximately 1.75 units. The observed fall in α-amino pK with increasing side-chain bulk (Table IV) follows the proposed pattern. Compensatory factors would likely reduce the total free-energy gain relative to this limiting case, but a quantitative apportionment of the free-energy balance to specific enthalpic or entropic terms would be difficult.

Apart from the crystallographic structural information, this interpretation turns on the significance of the stabilization of the protein attributable to the invariant residue Leu-2. The results of DiMarchi et al. (1979) showed substantial loss of stability and altered conformation upon removal of the second residue of the myoglobin molecule. Carbamylation of the newly exposed amino terminus did not compensate for this destabilization and thus discounts the possibility that relocation of the positive charge to Ser-3 causes these effects. It was concluded that the contributions of Leu-2 to stabilization of the myoglobin molecule were in large part dependent on the hydrophobic interactons of its side chain. A hydrophobic cluster arising from contact between the invariant residues Leu-2 and Leu-137 as seen in the crystal structure (Takano, 1977) would account for this stabilization and could also allow interaction of an aliphatic amino-terminal side chain to extend that cluster.

The crystal structure of harbor seal (*Phoca vitulina*) myoglobin (Scouloudi & Baker, 1978; Scouloudi, 1978) was also examined for evidence of the proposed hydrophobic cluster. The seal structure shows a reorientation of the terminal glycine, which removes the close contact of residues Leu-2 and Leu-137 but which allows close contact of the side chain of Leu-2 with the aliphatic portion of the Lys-133 side chain. In all, 30 Å^2 of Leu-2 side-chain area remain accessible in the seal structure that would be buried in the sperm whale structure. Leu-137 is similarly more exposed in the seal structure. Nevertheless, a substantial portion of the 164-Å^2 potential contribution of the Leu-2 side chain is removed from contact with solvent.

Dynamic Behavior of the Terminal Residue. The entropic consequences of the free-energy gain of the proposed hydrophobic cluster interaction were investigated by use of proton-decoupled ^{13}C NMR relaxation experiments. T_1 (spin–lattice relaxation time) and NOE (nuclear Overhauser enhancement) values were determined for the enriched carbons of each semisynthetic myoglobin product under varied conditions of pH for the analysis of the pH dependence of motions. Dipolar relaxation was taken as the predominant mechanism for all enriched carbons with the exception of the carbonyl carbons (Wittebort et al., 1979; Wilbur & Allerhand, 1977). The data were analyzed by the model-free approach of Lipari & Szabo (1982a,b) and can be applied to various models of molecular motions.

The Lipari-Szabo model-free approach to the analysis of NMR relaxation data involves calculation of two parameters to describe the phenomena: S, a generalized order parameter that increases with restriction of the motion, and τ_e, an effective correlation time, which is a measure of the rate of motion. These parameters determined for the α-carbon of each variant with varying pH are presented in Table VI. Values of τ_e determined for the α-carbon resonances are minimally 2 orders of magnitude lower than the 30-ns estimated lifetime of a proton on an α-amino group (Applegate et al., 1968). It is expected, therefore, that the protonation–deprotonation transition states do not significantly control the derived values.

Motions of the enriched terminal α-carbon of the [Gly^1]-myoglobin are clearly responsive to pH. Relaxation rates can

Table VI: α-Carbon Motions

	pH	T_1	NOE	S	τ_e (ps)
[Gly^1]myoglobin	6.61	0.56	1.03	0.73 ± 0.11[a]	11.8[b]
	7.25	0.60	1.00	0.70 ± 0.11	9.9
	8.30	0.77	1.12	0.60 ± 0.09	6.5
	8.88	0.91	1.03	0.56 ± 0.08	4.9
	9.59	0.92	1.01	0.56 ± 0.08	4.8
[Ala^1]myoglobin	6.2	0.36	1.35	0.73 ± 0.12	28.2
	7.5	0.50	1.87	0.52 ± 0.09	50.7
	9.1	0.43	1.84	0.56 ± 0.10	61.3
[Val^1]myoglobin	6.0	0.25	1.42	0.84 ± 0.12	91
	7.1	0.26	1.40	0.91 ± 0.12	163
	9.0	0.25	1.57	0.84 ± 0.09	157
[Ile^1]myoglobin	6.0	0.26	1.55	0.73 ± 0.10	124
	7.5	0.24	1.99	0.68 ± 0.09	84
	8.9	0.28	1.51	0.79 ± 0.10	186
[Leu^1]myoglobin	6.0	0.25	1.64	0.82 ± 0.14	84
	6.9	0.27	1.44	0.92 ± 0.14	209
	8.8	0.25	1.69	0.85 ± 0.14	91

[a] Values are reported as S ± an estimated maximum limit of error. These limits were calculated with error limits of ±10% for T_1 values and ±0.1 for NOE values. [b] Values are not specifically interpretable without the framework of a particular model. They are included only for relative comparisons within the effective correlation time scale. Error limits are difficult to estimate due to proximity to discontinuities in the function.

be plotted as a function of pH and fit to the Henderson–Hasselbalch equation with a pK of about 7.6 (Neireiter, 1979; Gurd et al., 1982). The order parameter shows motion decreasing as the pH changes from pH 9.6 to pH 6.6 (Table VI). The protonation of the terminal amino group will intensify electrostatic fields to induce additional organization of the surrounding water dipoles in a hydration shell, resulting in a restriction of motion. The order parameter determined for the α-carbon of [Ala^1]myoglobin shows a similar pH effect, but the effective correlation time, τ_e, is observed to be substantially larger relative to the [Gly^1]myoglobin case.

A similar effect can be seen in the [Gly^1]-terminal pentapeptide discussed earlier as a model compound (Neireiter, 1979). A substantial reduction of the T_1 value of the α-carbon from 1230 to 750 ms is seen upon protonation, indicative of a restriction of reorientational freedom and more effective relaxation. This can similarly be explained by the formation of an electrostatically organized hydration layer. A strikingly similar effect of protonation on T_1 values has been noted by Keim et al. (1973a,b) in studies of a series of pentapeptides with glycine-terminal residues.

In marked contrast with the behavior seen for the glycyl- and alanyl-terminal myoglobin variants, the α-carbons of the larger terminal residues of valine, isoleucine, and leucine have T_1 values (Table VI) that approach those determined for the α-carbon envelopes of the proteins, for which $T_1 \sim 0.22$ and $\tau_M \sim 9$ ns (Table VI). These examples do not reflect the pH sensitivity of the alanyl and glycyl variants.[5] The approach to the limiting case of the overall tumbling behavior defines a restriction of motion of the terminal α-carbon comparable to or exceeding that which results from the hydration phenomenon seen in the cases of the [Gly^1]- and [Ala^1]myoglobins in which the α-amino group is protonated. The trend is most apparent in the T_1 values in Table IV. It is also reflected in the trend of the computed order parameters, which, however, lose sensitivity as the limiting value of unity is approached.

The suitability of the terminal side chain to extend a hydrophobic cluster arising from contact between the invariant residues Leu-2 and Leu-137 seen in the crystal structure (Takano, 1977), as discussed above, could be the basis for the differences in dynamic behavior between the different variants. The proposed reduction in motional freedom by energetically favorable hydrophobic clustering can be further supported by analysis of isoleucine side-chain behavior (M. R. Busch, unpublished observations). The terminal isoleucine shows restriction of the C_α–C_β and C_β–C_{γ_1} bond rotations similar to those seen for 8 internal isoleucines (Wittebort et al., 1979) when the slight additional contribution of the terminal α-carbon motion is taken into account. This behavior, similar to that of internal isoleucines where packing densities are high, would be consistent with participation in a hydrophobic cluster.

The significance of these dynamic observations in analysis of the behavior seen for the pK values lies in the nature of the hydration of the protein. Proteins can contain irrotationally bound water molecules, but the bulk of associated water is under weak orientational constraint from the surface and tumbles less than 1 order of magnitude more slowly than bulk solvent (Halle et al., 1982). Charged groups will induce larger electrostatically organized perturbations in this hydration layer (Watenpaugh et al., 1978; Blake et al., 1983; Matthew, 1985).

The ability of the terminal glycine to respond to the protonation event by moving into the bulk solvent to enlarge its hydration volume and the restricted ability of the larger side chains to respond in a similar manner account for the behavior of the pK values. Restriction of the time-average size of the hydration volume, by rendering the process of protonation energetically less favorable, will produce the observed trend of the pK values. The free-energy gain of hydrophobic stabilization that accompanies energetically favorable hydrophobic clustering as the more bulky aliphatic side chains condense on the protein surface is balanced by the expense of the restriction of mobility and of the altered protonation equilibrium.

Effects of Single-Site Substitutions. This study of the consequences of systematic single-site variations in proteins shows that the interpretation of the roles of a residue is facilitated by a combination of modes of observation. It illustrates an approach to the understanding of the role of the usually "silent" aliphatic amino acids, made possible by the combination of motional analysis with measurement of the pK of the amino group of the residue.

In energetic terms, the progressive depression of the amino-terminal pK value in this series absorbs a substantial fraction of the calculated free-energy gain as the hydrophobic interaction of the residue increases. These changes are reflected in the dynamic behavior, but the possibility of minor conformational adjustments of the A helix to accommodate the side-chain alterations and resulting packing constraints will be substantially more difficult to define in terms of their energetic compensation (DiMarchi et al., 1980).

The approach of the observed dynamic behavior to the values imposed by tumbling of the protein molecule suggests that the range of hydrophobic interaction stability accessible to a single aliphatic residue is effectively represented by this series of variants. Thus, the sensitivity of methods for probing individual site contributions of hydrophobic interactions must be comparable to that attained in this study. The selection of useful variations in the engineering of proteins by semisynthetic or mutagenic techniques requires a prior knowledge

[5] Approach of the relaxation parameters of the α-carbons to those observed for the overall tumbling of the protein makes it more difficult to distinguish the internal motions of the enriched α-carbons from the overall reorientation process of the protein. This results in a loss of accuracy in the calculated S and τ_e parameters (Lipari & Szabo, 1982b). Therefore, the superimposed effect of pH on a substantially restricted motion may be more difficult to detect.

or awareness of the subtleties involved in the integrated contributions of a single residue to the functional form of a protein structure. By detection of effects of single-site changes in an appropriate energy range, this study represents significant progress toward the understanding necessary for informed choices in protein engineering.

ACKNOWLEDGMENTS

We gratefully acknowledge the generous gift of ^{13}C-labeled amino acids from Dr. N. A. Matwiyoff and Dr. R. E. London of Los Alamos National Laboratory. The technical assistance of Kenney Green and Leigh Ann Eriks was invaluable, as was that of Robert Addleman and Deon Osman. Bertrand Garcia-Moreno E. provided significant insight during numerous discussions. Drs. Attila Szabo, Adam Allerhand, and Helen Scouloudi provided advice and encouragement. Dr. R. J. Wittebort is thanked for careful review of the manuscript. Sarah Burton was invaluable in the typing and preparation of the manuscript.

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