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Structural Significance of the Amino-Terminal Residues of Sperm Whale Myoglobin†

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ABSTRACT: Following the development of a nondestructive synthetic procedure for rapid production of des-Val[1]-myoglobin in large quantities, the synthesis of a series of myoglobin derivatives varying in structure and charge in the NH_2-terminal region was accomplished. In comparison to the untreated myoglobin, the des-Val[1]-myoglobin was found to possess at low pH a decreased stability and an increased net positive charge in the pH range 5.5–8.5. While the elevated net positive charge was no longer apparent after removal of the second residue, the instability of the molecule was found to be sharply increased. Substitutions of the first residue, directed toward elucidating its structural importance, included glutamic acid, lysine, and glycine. Addition of any of the three amino acids to the des-Val[1]-myoglobin was found to restore much of the acid stability, with the [Gly[1]]myoglobin appearing nearly identical with the native molecule. All three semisynthetic myoglobins showed potentiometric titration curves characteristic of their respective, substituted residue. Carbamylation of the NH_2 terminal of myoglobin and des-Val[1]-myoglobin yielded two nearly identical molecules in terms of all physical properties examined. Consequently, it was concluded that the first residue primarily serves the function of maintaining the positively charged NH_2 terminus a certain distance away from the beginning of the A helix and from the charge pair interaction of Lys-133 with Glu-6. In addition, through physical measurements of the des-Val[1],Leu[2]-myoglobin prior and subsequent to carbamylation of the NH_2 terminus, it was apparent that the stabilization conferred on the des-Val[1]-myoglobin by the second residue was dependent to a large degree upon the hydrophobic interactions of its side chain.

Previous studies (Wang, 1977; Wang et al., 1978) have revealed that removal of the NH_2-terminal tetradecapeptide of myoglobin results in a molecule possessing less than half of the normal α-helical content and incapable of correctly positioning the heme. Which specific residue or residues are responsible for this loss of structure and through what mechanism they interact with those residues constituting the heme pocket are central to understanding the stabilizing forces of the molecule. Recent studies (DiMarchi et al., 1978b, 1979; Neireiter et al., 1979) have provided the necessary techniques for selective, consecutive removal and substitution of the NH_2-terminal residues of myoglobin by specific degradation and resynthesis. The application of these techniques can make apparent the structural significance of each amino acid residue and show the effects of various substitutions and modifications with respect to stability, conformation, and electrostatic and hydrophobic interactions.

Crystallographic studies of sperm whale aquoferrimyoglobin (Watson, 1969; Takano, 1977) have shown that the NH_2-terminal residues lie predominantly on the surface of the molecule with no direct interactions with those residues exhibiting short interatomic contacts (4.0 Å or less) with the heme. The NH_2-terminal valine residue is predicted to exhibit limited electrostatic interactions with the remaining polypeptide (Friend & Gurd, 1979a,b; Matthew et al., 1979), and both it and the second residue possess no regular, ordered secondary structure (Chou & Fasman, 1974; Takano, 1977). Consequently, the finding of only minor changes in the molecule following NH_2-terminal adduct formation was not surprising (Garner & Gurd, 1975; Gurd et al., 1977; DiMarchi et al., 1979). Nonetheless, the highly conserved nature of the first two residues suggests some type of specific structural importance; glycine is the only other NH_2-terminal residue observed among more than 50 myoglobin species sequenced, while leucine in the second position is invariant (Bogardt, 1978).

Artyukh et al. (1977, 1979) have recently implicated the deprotonation of the NH_2 terminus as a conformational trigger for the A helix and implied that this change is responsible for a perturbation in the spin equilibrium of the heme iron atom. The importance of these findings is emphasized by the known importance of the hemoglobin NH_2 terminals in regulating control of ligand binding (Kilmartin & Rossi-Bernardi, 1969; Benesch et al., 1969; Perutz, 1970; Garner et al., 1975; Matthew et al., 1977). Crystallographic analyses of the hydroxide, cyanide, azide, and fluoride complexes of sperm whale ferrimyoglobin have shown varying degrees of conformational changes in certain regions of the protein, including residues 62–75 of the E helix (Stryer et al., 1964; Watson & Chance, 1966; Bretscher, 1968; Schoenborn, 1969). While no connection had been drawn hitherto between conformational changes and the protonation state of the NH_2 terminus, it is interesting to note the additional minor alterations in several residues involved in important charge interactions neighboring the NH_2 terminus (Schoenborn, 1969; Friend & Gurd, 1979a,b).

The results of the present study clearly illustrate the delicate balance which exists in the myoglobin molecule between the structure of the NH_2-terminal residues and the conformation

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and stability of the remainder of the protein. Removal of the NH_2-terminal valine residue yielded a myoglobin less stable to acid and showing some special characteristics on titration (Breslow & Gurd, 1962; Hartzell et al., 1968). A sizable alteration in molecular conformation was observed for the des-Val[1],Leu[2]-myoglogin.[1] Changes were observed in the ability to reassociate properly with hemin, the tryptophan circular dichroism absorbance, and the heme iron spin equilibrium. In an attempt to ascertain the mechanism of perturbation occurring in these shortened myoglobins, we modified the charge and structure at the NH_2-terminal region through carbamylation, maleylation, and preparation of three semisynthetic derivatives, [Glu[1]]-, [Lys[1]]-, and [Gly[1]]myoglobins.

Experimental Section

The isolation and purification of the principal component myoglobin of sperm whale were carried out as previously described (Hapner et al., 1968). The principal component was used as the starting material for all studies. Boc-Glu(OBu^t) ester and Lys(CF_3CO) were purchased from Vega-Fox Chemical Co. Sodium dithionite and potassium ferricyanide were obtained from J. T. Baker while the sources of all other chemicals were previously reported (DiMarchi et al., 1979).

The experimental methods used in amino acid analysis, potentiometric hydrogen ion titrations, cellulose acetate electrophoresis, circular dichroism, and ultraviolet–visible absorbance measurements are described elsewhere (DiMarchi et al., 1978b, 1979). The preparation of the acetimidomyoglobin and its reaction with H_4Pht-anhydride followed the procedure of DiMarchi et al. (1978b). Histidine proton nuclear magnetic resonance spectra were obtained as described by Botelho & Gurd (1978). The hemic acid dissociation constant was measured by the procedure of George & Hanania (1952) as adapted by Nakhleh (1971). Attempts to remove the Leu-2 residue from the des-Val[1]-N^{ϵ}_{19}-acetimidomyoglobin with leucine aminopeptidase followed the general procedure of Light (1972).

Acid Stability Measurements. An aliquot of an aquoferrimyoglobin derivative was added to a 0.1 M citrate buffer at a predetermined pH value, 25 °C, and the 409-nm absorbance was recorded after equilibration. The fraction of protein remaining native, at each pH value, was calculated by subtracting the absorbance at pH 3.6 (completely denatured) from the absorbance at the predetermined pH and dividing this quantity by the difference between the absorbances at pH 5.8 (completely native) and pH 3.6 (Theorell & Ehrenberg, 1951; Hartzell et al., 1968; Puett, 1973; Friend & Gurd, 1979a).

Reaction of Maleic Anhydride with Acetimidomyoglobin. A 3% solution of acetimidated ferrimyoglobin was allowed to react with a 10-fold excess of maleic anhydride at pH 7.2 ± 0.2, 15 °C, for 30 min. The pH was maintained by the addition of 0.5 N NaOH. Upon completion of reaction, the protein was dialyzed for 24 h against water at 4 °C (Butler et al., 1969).

Reaction of Potassium Cyanate with Acetimidomyoglobin. To a 2% solution of acetimidated cyanoferrimyoglobin (2.0 g) in a pH 6.5, 0.01 M phosphate buffer at 25 °C was added a 180-fold molar excess of KNCO (Stark et al., 1960). After 1 h of carbamylation, the pH was adjusted to 7.4 at 15 °C and a 10-fold excess of H_4Pht-anhydride (170 mg) was added. All free amines were found reacted within a period of 1 h. The cyanoferrimyoglobin was deionized on a Rexyn I-300 column (1.5 × 27 cm). For displacement of the cyanide ligand, the protein was reduced to the ferro form in the presence of a 10-fold excess of sodium dithionite for 30 min, after which time it was deionized once more and oxidized to the aquoferri form with a 10-fold excess of potassium ferricyanide (Garner et al., 1975).

Reaction of 3-Sulfophenyl Isothiocyanate with Acetimidoferromyoglobin. The experimental details for the preparation of the acetimidoferrimyoglobin have been previously described (DiMarchi et al., 1978a,b, 1979). If the relative proportion of N^{ϵ}_{19}-acetimidomyoglobin was found to be less than 55% by electrophoresis following reaction with H_4Pht-anhydride (DiMarchi et al., 1979), the protein was subjected to the acetimidation conditions once more. Without purification of the acetimidation products, a 1-g aliquot in 5% solution was reduced to the ferro form by passage through a previously loaded 15-fold excess of sodium dithionite on a Bio-Gel P-2 column (2.7 × 42 cm) in 0.02 M phosphate buffer (carbon monoxide saturated), pH 7.2, 25 °C. Upon elution, the acetimidoferromyoglobin was immediately converted to the carbon monoxy form by passing a stream of CO over the stirred surface of the protein solution. The acetimido(carbon monoxy)myoglobin (25 mg/mL) was treated with a 10-fold excess of 3-sulfo-PhNCS at pH 8.0, 25 °C, for 30 min under a nitrogen atmosphere (Neireiter et al., 1979). Upon completion of reaction, the products were desalted on a Rexyn I-300 column (2.3 × 20 cm) in nitrogen-saturated deionized water. Oxidation of the ferrous heme was accomplished by addition of a 15-fold excess of potassium ferricyanide to the stirred protein at 25 °C for 10 min. The protein was desalted once more, prior to removal of the heme, on a Rexyn I-300 column (2.3 × 20 cm) in nitrogen-saturated water.

Removal of the NH_2-Terminal Residue. The N^{α}-3-sulfo-PhNHCS-acetimidoferrimyoglobin was made 10 mM in NaF, and the pH was adjusted to 3.5 at 2 °C with 1 N HCl (Yonetani, 1967; DiMarchi et al., 1979). The heme was extracted with ice-cold 2-butanone and the apoprotein was lyophilized immediately. Cleavage of the 3-sulfo-PhNHCS-valine was accomplished as previously described (DiMarchi et al., 1979), using 200 mg of dithioerythritol, 100 μL of anisole, and 10 g of trifluoroacetic acid per g of apoprotein. Following removal of all the trifluoroacetic acid, the des-Val[1]-acetimidoapomyoglobin was rehydrated to a concentration of 10 mg/mL and dialyzed against water at 2 °C until a pH of ~5.5 was obtained.

Reconstitution of the Des-Val[1]-acetimidomyoglobin with Hemin. The stoichiometric amount of hemin was determined by adding increasing amounts of hemin chloride to several samples of apoprotein (3 mg/mL) in 0.01 N sodium hydroxide until a maximum value of the 409 nm/280 nm absorbance ratio was obtained. After 15 min at 25 °C, the pH of the reconstitution reaction was lowered by dilution to 5 mL with μ = 0.1 phosphate buffer, pH 6.5, for determination of the absorbance ratio.

To a 1% solution of the acetimidoapomyoglobin adjusted to pH 10.8 at 4 °C was added dropwise a stoichiometric amount of hemin chloride dissolved in a minimum amount of 0.1 N sodium hydroxide. After 30 min of reconstitution at pH 10.8, 4 °C, the des-Val[1]-acetimidomyoglobin was dialyzed

[1] Abbreviations used: At, acetimidyl; des-Val[1]-, myoglobin which lacks the NH_2-terminal residue; des-Val[1],Leu[2]-, myoglobin which lacks the NH_2-terminal dipeptide valylleucine; H_4Pht, 3,4,5,6-tetrahydrophthalyl; $HO_2CCH{=}CHCO$, maleyl; Boc, *tert*-butoxycarbonyl; NH_2CO, carbamoyl; CF_3CO, trifluoroacetyl; OBu^t, *tert*-butyl ester; ONSu, *N*-hydroxysuccinimide ester; 3-sulfo-PhNHCS, (3-sulfophenyl)thiocarbamoyl; 3-sulfo-PhNCS, 3-sulfophenyl isothiocyanate; NCO, cyanate; N^{α}, modification at the NH_2-terminal nitrogen atom; N^{ϵ}_{19}, modification at the ε-amino atom of all 19 lysine residues.

extensively against water. Improperly reconstituted protein was precipitated as a hemochromogen through dialysis for 12 h against $\mu = 0.1$ phosphate buffer, pH 6.5, 4 °C, and removed by centrifugation (DiMarchi et al., 1979). The des-Val^1-N^{ϵ}_{19}-acetimidomyoglobin was isolated on a CM-Sephadex C-50 column (9.5 × 55 cm), through elution with $\mu = 0.1$ phosphate buffer, pH 6.45, at 25 °C.

Preparation of Des-Val^1,Leu^2-N^{ϵ}_{19}-acetimidomyoglobin. The des-Val^1-N^{ϵ}_{19}-acetimidomyoglobin was recycled through the dithionite reduction, 3-sulfo-PhNCS coupling for 60 min, ferricyanide oxidation, heme removal, trifluoroacetic acid cleavage, and heme reconstitution as described in the synthesis of the des-Val^1-N^{ϵ}_{19}-acetimidomyoglobin to prepare the des-Val^1,Leu^2-N^{ϵ}_{19}-acetimidomyoglobin. The protein was purified on a CM-Sephadex C-50 column (1.7 × 25 cm) in $\mu = 0.1$ phosphate buffer, pH 6.5, 25 °C.

Removal of the Acetimidyl Group. The acetimidyl groups were removed by exposure of the holoprotein derivative to concentrated ammonium hydroxide–acetic acid (30:1 v/v) at pH 11.8, 25 °C (Ludwig & Byrne, 1962; Reynolds, 1968), for 24 h. After ammonolysis, the deacetimidated protein was exhaustively dialyzed at 4 °C against water, and the content of lysine and acetimidolysine was determined through 24-, 48-, and 72-h hydrolysates in 5.7 N HCl (Spackman et al., 1958; DiMarchi et al., 1978b) or, preferably, through *p*-toluenesulfonic acid hydrolysis (Liu & Chang, 1971; Neireiter, 1979). All deacetimidated derivatives were purified on CM-Sephadex C-50 in $\mu = 0.1$ phosphate buffer.

Direct Preparation of Des-Val^1-myoglobin. To 1 g of (carbon monoxy)ferromyoglobin, prepared as described earlier with the acetimidomyoglobin, was added a 10-fold excess of 3-sulfo-PhNCS at pH 7.0, 25 °C. After reaction for 60 min, the removal of the NH_2-terminal residue and subsequent heme reconstitution were effected as described earlier in the preparation of the des-Val^1-N^{ϵ}_{19}-acetimidomyoglobin. Des-Val^1-myoglobin was isolated on a CM-Sephadex C-50 column (9.5 × 47 cm) through elution with $\mu = 0.1$ phosphate buffer, pH 6.45, in 8% yield.

Synthesis of [Glu^1]myoglobin. The *N*-hydroxysuccinimide ester of Boc-Glu(OBu^t) ester was synthesized following the procedure of Anderson et al. (1964). Thin-layer chromatography in 60:30:1:4 (chloroform–methanol–water–acetic acid) revealed only one spot with an R_f value of 0.90: yield, 83%; mp 140–141 °C. The absence of any racemization was confirmed through separation of any diastereoisomeric dipeptides with DL-leucine (Manning & Moore, 1968; Mitchell et al., 1978).

The *N*-hydroxysuccinimide ester of Boc-Glu(OBu^t) ester (320 mg) was added in 20-fold excess to a 2% solution of des-Val^1-N^{ϵ}_{19}-acetimidomyoglobin (700 mg) in 30% methanol–water. After 18 h of reaction at an apparent pH of 7.8 ± 0.1, 17 °C, the protein was dialyzed against water for 12 h at 2 °C. The N^{α}-Boc-Glu^1(OBu^t)-acetimidomyoglobin was purified on a CM-Sephadex C-50 column (3 × 50 cm) in $\mu = 0.1$ phosphate buffer, pH 6.35. After removal of the heme (DiMarchi et al., 1979), cleavage of the Boc and OBu^t ester was accomplished by trifluoroacetic acid treatment for 60 min as previously described (Kusch, 1966; DiMarchi et al., 1979). The impure [Glu^1]-N^{ϵ}_{19}-acetimidomyoglobin was reconstituted with hemin as described earlier for the des-Val^1-N^{ϵ}_{19}-acetimidomyoglobin and purified on a CM-Sephadex C-50 column (2.2 × 32 cm) at pH 6.3 in $\mu = 0.1$ phosphate buffer. Following removal of the acetimidyl protecting groups, the [Glu^1]myoglobin was purified on a CM-Sephadex C-50 column (2.5 × 37 cm) at pH 6.0 in $\mu = 0.1$ phosphate buffer. The synthetic yield starting from the coupling step with the activated ester was 12%.

Synthesis of [Lys^1]myoglobin. Boc-Lys(CF_3CO) was synthesized following the procedure of Itoh et al. (1975) and crystallized by the method of Anfinsen et al. (1967): yield, 55%; mp 101–103 °C; R_f value of 0.38 in 60:30:1:4 (chloroform–methanol–water–acetic acid). The *N*-hydroxysuccinimide ester of Boc-Lys(CF_3CO) was prepared by the procedure of Wolman & Klausner (1971): yield, 62%; mp 117–119 °C. Due to the greater solubility, in 30% methanol, of the lysine-ONSu ester in comparison to that of the glutamic acid derivative, coupling to the des-Val^1-acetimidomyoglobin was performed for only 5 h. Following dialysis, purification of the N^{α}-Boc-Lys^1(CF_3CO)-acetimidomyoglobin, removal of the Boc group, and reincorporation of the heme, the amino protecting groups were removed concomitantly (DiMarchi et al., 1979). The [Lys^1]myoglobin was purified on a CM-Sephadex C-50 column (5 × 60 cm) in a linear gradient of $\mu = 0.1$ phosphate buffer, pH 6.4–6.6. The synthetic yield starting from the coupling step with the activated ester was 24%.

Synthesis of [Gly^1]myoglobin. CF_3CO-Gly-ONSu was prepared by Neireiter (1979) following the procedure of DiMarchi et al. (1978a): yield, 32%; mp 135–137 °C. Due to the increased water solubility of the glycine active ester, coupling to des-Val^1-acetimidomyoglobin was performed in water at a 10-fold excess, pH 7.5, 15 °C, for 2 h. The amino protecting groups were removed in one step (DiMarchi et al., 1978a; Neireiter, 1979), and the protein was purified on a CM-Sephadex C-50 column (6.6 × 54 cm) in $\mu = 0.1$ phosphate buffer, pH 6.45. A small impurity detected by electrophoresis at pH 9.2 was removed by further purification of the [Gly^1]myoglobin on a DEAE-Sephadex A-50 column (4.5 × 60 cm) in 0.05 M Tris buffer, pH 9.1, at 2 °C (Neireiter, 1979). The synthetic yield starting from the coupling step with the activated ester was 29%.

Results

Figure 1 illustrates the synthetic procedure utilized in removing the NH_2-terminal residues from acetimidomyoglobin. This strategy represents a significant improvement in synthetic yield and time for synthesis of des-Val^1-N^{ϵ}_{19}-acetimidomyoglobin (DiMarchi et al., 1979).

3-Sulfo-PhNCS Coupling to Acetimidoferromyoglobin. Conversion of the acetimidoferrimyoglobin to the ferro form was found to prevent 3-sulfo-PhNCS binding to the heme (DiMarchi, 1978) and the possible heme-catalyzed oxidation of the resultant NH_2-terminal thiourea (Ilse & Edman, 1963). Consequently, it eliminated the fundamental reasons for employing the apoprotein (DiMarchi et al., 1979). Essentially quantitative reaction of the 3-sulfo-PhNCS with the NH_2 terminus (Neireiter et al., 1979) yielded reaction products which were easily identified by electrophoresis (Figure 2D). Through analogy with the electrophoretic separation of the H_4Pht-anhydride reaction with acetimidomyoglobin (Figure 2C), the component found moving with one additional net negative charge is predominantly N^{α}-3-sulfo-PhNHCS-N^{ϵ}_{19}-acetimidomyoglobin. The other sizable component represents reaction at an unprotected ϵ-amino group in addition to the NH_2 terminus.

Removal of the Heme. Following reaction with 3-sulfo-PhNCS, the only method found capable of quantitatively removing the heme required ferricyanide oxidation of the ferro form to the ferri state, prior to 2-butanone extraction at pH 3.5 in the presence of 10 mM NaF. For protein precipitation to be avoided, it was found imperative to remove the excess 3-sulfo-PhNCS and phosphate buffer before oxidation of the

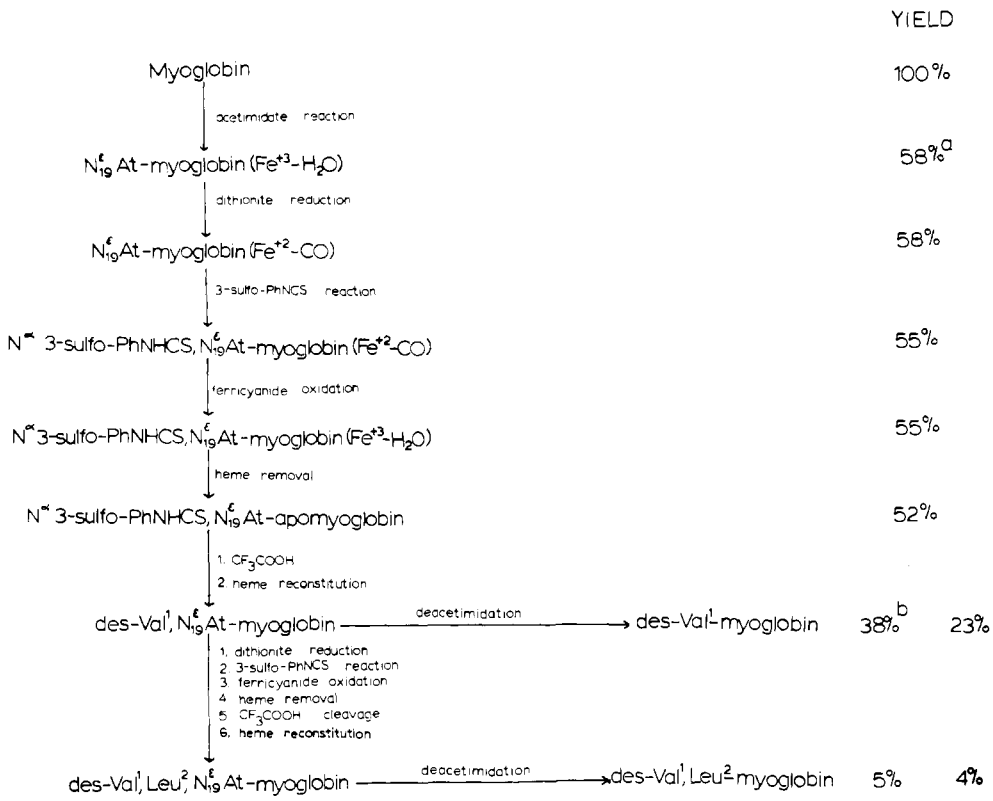

FIGURE 1: Flow chart outlining the synthesis of des-Val[1]-myoglobin and des-Val[1],Leu[2]-myoglobin. (a) The yield given is obtained from electrophoretic data as given in Figure 2 and does not represent its actual isolation. This is true also of the next four synthetic intermediates. (b) The yield is given after purification, prior to H_4Pht-anhydride removal of material with less than complete lysine acetimidation.

heme and the excess ferricyanide prior to heme extraction. The dissolved 2-butanone was removed in the lyophilization step rather than through dialysis, in order to minimize oxidation of the N^{α}-3-sulfo-PhNHCS substituent. The presence of 10 mM NaF throughout the trifluoroacetic acid cleavage had no noticeable effects (see paragraph at end of paper regarding supplementary material), while higher levels led to formation of an anomalous component which appeared more positive than all other components.

Trifluoroacetic Acid Cleavage of the N^{α}-3-Sulfo-PhNHCS-Valine. Since N^{ϵ}-3-sulfo-PhNHCS-lysine formed by reaction with any unprotected ϵ-amino groups would be stable in anhydrous trifluoroacetic acid (Edman & Henschen, 1975), the cleavage of the N^{α}-3-sulfo-PhNHCS derivative of valine (DiMarchi et al., 1979) returns only the des-Val[1]-acetimidomyoglobin to a point isoelectric with the N^{ϵ}_{19}-acetimidomyoglobin (Figure 2B,E). In this way the procedure employed in the synthesis of the des-Val[1]-N^{ϵ}_{19}-acetimidomyoglobin (Figure 1) also served to distinguish the desired component from all other contaminants. Consequently, the production of des-Val[1]-N^{ϵ}_{19}-acetimidomyoglobin from myoglobin was achieved in 38% yield with just one purification step (Figure 3A). However, electrophoretic analysis (Figure 2G) of the des-Val[1]-N^{ϵ}_{19}-acetimidomyoglobin after reaction with H_4Pht-anhydride reveals 20% total contaminants of N^{ϵ}_{18}-acetimidomyoglobin,[2] which were removed prior to any physical studies (DiMarchi et al., 1979). The possibility of a small amount of HF generated in situ during the cleavage step (due to the presence of NaF) causing the 1% deacetimidation was eliminated with appropriate control experiments (see supplementary material). Apparently the trifluoroacetic acid is responsible for a slightly higher amount of deacetimidation than previously observed (DiMarchi et al., 1979). The des-Val[1]-myoglobin was obtained through deacetimidation and, following purification (Figures 2J,K and 3B), electrophoresis revealed it to comigrate with untreated myoglobin at pH 9.2 (Figure 2K,N). NH_2-Terminal sequence analysis of the des-Val[1]-myoglobin revealed only leucine in the first cycle, as shown in Table I.

Synthesis of Des-Val[1],Leu[2]-myoglobin. Subjection of the des-Val[1]-N^{ϵ}_{19}-acetimidomyoglobin to the degradative cycle illustrated in Figure 1 provided the des-Val[1],Leu[2]-N^{ϵ}_{19}-acetimidomyoglobin in a surprisingly low yield of 15%. Apparently the twice-shortened apoacetimidomyoglobin does not reassociate well with hemin, as the maximum 409 nm/280 nm absorbance ratio obtained through several trials was only 2.96. Consequently, the majority of the protein precipitated as hemochromogen and the des-Val[1],Leu[2]-N^{ϵ}_{19}-acetimidomyoglobin was purified from the soluble fraction by ion-exchange chromatography (Figure 3C). Following removal of the acetimidyl groups and purification (Figure 3D), analysis of the NH_2-terminal residue of the des-Val[1],Leu[2]-myoglobin revealed no leucine in the first cycle and only glutamic acid in the second cycle (Table I). The purified des-Val[1],Leu[2]-myoglobin was found to have a 409 nm/280 nm absorbance ratio of 4.95 (Table II).

Maleylation and Carbamylation of Acetimidomyoglobins. The ability to maleylate or carbamylate the α-amino group selectively provided the opportunity to assess the importance of a positively charged NH_2 terminus. Maleic anhydride was found to react quantitatively with all free amino groups in both the N^{ϵ}_{19}-acetimidomyoglobin (Figure 4C,D) and the des-Val[1]-N^{ϵ}_{19}-acetimidomyoglobin, yielding a profile of products

[2] The absence of any free amines in the 3-sulfo-PhNCS-coupled acetimidomyoglobin (Figure 2D) was confirmed by the absence of reaction with H_4Pht-anhydride. The smaller contaminant after trifluoroacetic acid treatment (Figure 2G) represents most probably deacetimidation at a lysine residue bearing a pK value lower than 9.2, presumably Lys-77 (DiMarchi et al., 1978a,b).

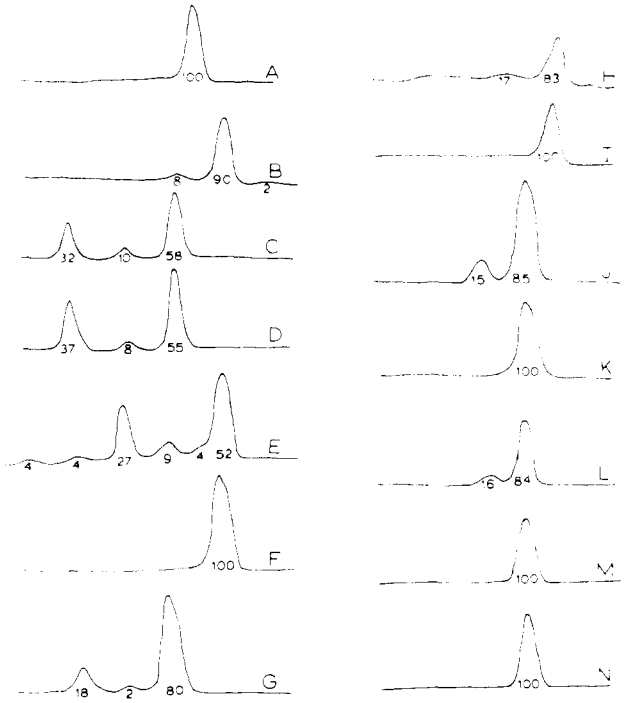

FIGURE 2: Densitometer integration of the electrophoretic separation of the untreated myoglobin and acetimidomyoglobin derivatives at pH 9.2, 300 V, in 0.1 M Tris–EDTA–boric acid. (A) Untreated myoglobin; (B) products of myoglobin acetimidation reaction, predominantly N^{ϵ}_{19}-acetimidomyoglobin; (C) H_4Pht-anhydride reaction of acetimidomyoglobin products shown in (B); (D) 3-sulfo-PhNCS reaction of acetimidomyoglobin products shown in (B); (E) impure des-Val^1-acetimidomyoglobin, as obtained following trifluoroacetic acid cleavage and heme reconstitution; (F) des-Val^1-acetimidomyoglobin; (G) H_4Pht-anhydride reaction of des-Val^1-acetimidomyoglobin; (H) impure des-Val^1,Leu^2-acetimidomyoglobin; (I) des-Val^1,Leu^2-acetimidomyoglobin; (J) impure des-Val^1-myoglobin following deacetimidation; (K) des-Val^1-myoglobin; (L) impure des-Val^1,Leu^2-myoglobin following deacetimidation; (M) des-Val^1,Leu^2-myoglobin; (N) untreated myoglobin.

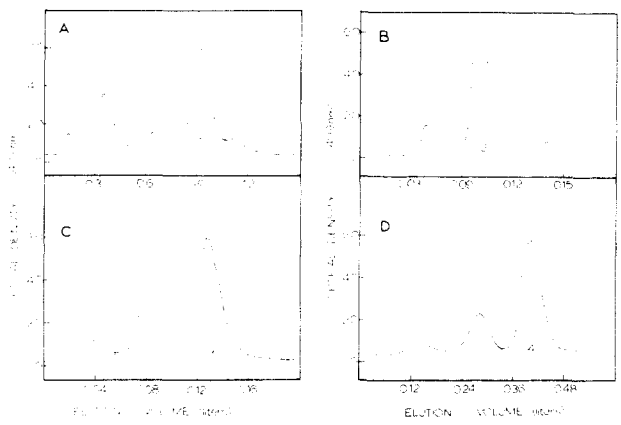

FIGURE 3: Elution profiles of chemically modified myoglobins. All purifications utilized CM-Sephadex C-50 resin with μ = 0.1 phosphate as the eluting buffer. (A) Purification of des-Val^1-N^{ϵ}_{19}-acetimidomyoglobin (peak 1) on a column (9.5 × 55 cm), at pH 6.45, 25 °C, and 165 mL/h, following acetimidate reaction, 3-sulfo-PhNCS reaction, heme removal, CF_3COOH cleavage, and heme reconstitution; (B) isolation of products following deacetimidation of des-Val^1-N^{ϵ}_{19}-acetimidomyoglobin on a column (2.3 × 30 cm), at pH 6.45, 25 °C, and 20 mL/h, with peak 2 representing des-Val^1-myoglobin; (C) purification of des-Val^1,Leu^2-N^{ϵ}_{19}-acetimidomyoglobin (peak 3) after subjection to each of the cleavage steps on a column (1.7 × 25 cm), at pH 6.5, 25 °C, and 22 mL/h; (D) isolation of products following diacetimidation of des-Val^1,Leu^2-N^{ϵ}_{19}-acetimidomyoglobin on a column (2.4 × 51 cm), at pH 6.45, 25 °C, 24 mL/h, with peak 4 representing des-Val^1,Leu^2-myoglobin.

nearly identical with that through reaction with H_4Pht-anhydride. The HC·CCH=CHCO substituent is known to be sufficiently stable to acid to ensure its presence throughout the acid stability and potentiometric titration measurements (Butler et al., 1969).

While the conditions used in carbamylation of the acetimidomyoglobins were found to be quantitative for the NH_2 terminus, less than complete reaction was noted with any free ϵ-amino groups (Figure 4I–K). Consequently, immediately following the carbamylation reaction, the impure protein products were treated with maleic anhydride or H_4Pht-anhydride to simplify the purification of the desired derivative. The N^{α}-NH_2CO-N^{ϵ}_{19}-acetimidomyoglobin, being incapable of reacting with the anhydride, remains the most positive component and is easy to purify (Figure 4K,L). Following deacetimidation no loss of either the maleyl or the carbamoyl group was noticeable, as determined by NH_2-terminal sequence analysis.

Semisynthetic Myoglobin Variants. Together with the native myoglobin the three semisynthetic derivatives [Glu^1]-, [Lys^1]-, and [Gly^1]myoglobin form a series of molecules varying in side-chain charge and size at the first residue. The coupling yields of the ONSu esters of glutamic acid, lysine, and glycine to the α-amino group were 69, 94, and 95%, respectively (Figure 5B,C,J–L,R–T). The lower degree of coupling observed with Boc-Glu(OBu^t)-ONSu results primarily from its lower water solubility (DiMarchi, 1978). In the case of the glutamic acid and lysine couplings, methanol in a concentration of 30% was necessary to maintain the active ester reasonably soluble. The selection of methanol as a co-solvent was based on the fact that myoglobin is readily denatured by most organic solvents (Herskovitz & Solli, 1975; Herskovitz et al., 1977) but appears to be stabilized by methanol in the concentration range of 25% (Asakura et al., 1978; DiMarchi, 1978). The higher reactivity of the glycine ester resulted in the apparent minor coupling to a side-chain residue (Figure 5R–T), presumably a tyrosine (DiMarchi et al., 1979; Martinez et al., 1979). The concomitant removal of the trifluoroacetyl and acetimidyl groups (Figure 5U) in concentrated alkali simplified the purification of the [Gly^1]-myoglobin (Neireiter, 1979). The Boc and OBu^t groups of the [Glu^1]- and [Lys^1]myoglobins were removed by subjecting the proteins once more to trifluoroacetic acid treatment. Dithioerythritol and anisole were found to be most appropriate scavengers for the *tert*-butyl cation and *tert*-butyl trifluoroacetate, as reported by Lundt et al. (1978). Following rehydration, both derivatives reassociated with hemin to yield 409 nm/280 nm absorbance ratios approaching that of the native protein. Subsequently, the semisynthetic variants were each purified on a cation-exchange column to yield one nearly homogeneous product (parts H and P, respectively, of Figure 5).

A surprising result encountered in the preparation of the [Lys^1]myoglobin was the apparent removal of the CF_3CO protecting group (Anfinsen et al., 1967) of the ϵ-amino function of the [Lys^1] residue before the alkaline deacetimidation step. The alkaline exposure during the reintroduction of the hemin proved to be sufficient to effect the removal of the CF_3CO group. The trifluoroacetic acid treatment itself probably did not cause the deprotection of the ϵ-amino group in question (Figure 5N,O).

Potentiometric Hydrogen Ion Titrations. The potentiometric titrations of seven myoglobin derivatives varying in structure at the NH_2-terminal residues are presented in Figure 6. For comparative purposes these derivatives have been separated for presentation into three convenient categories

Table 1: Amino Acid Composition[a] and NH_2-Terminal Analysis[b] of the Myoglobin Derivatives

		myoglobin derivatives				
amino acid	myoglobin	des-Val[1]	des-Val[1],Leu[2]	[Gly[1]]	[Glu[1]]	[Lys[1]]
Asp	8	8.3	7.9	8.1	8.1	8.1
Thr	5	4.9	5.0	5.0	4.9	5.1
Ser	6	5.8	6.0	5.9	5.7	5.9
Glu	19	19.2	19.1	19.0	20.2	19.2
Pro	4	3.8	4.1	4.1	4.0	3.9
Gly	11	11.3	11.1	12.1	11.4	11.2
Ala	17	17.2	17.1	16.9	17.3	17.2
Val	8	7.1	7.0	7.0	6.9	7.1
Met	2	2.0	1.9	1.9	1.9	1.9
Ile	9	8.8	9.1	9.1	9.0	9.3
Leu	18	18.3	17.1	18.2	18.3	18.4
Tyr	3	3.0	2.8	3.0	2.9	2.9
Phe	6	6.0	5.8	5.9	5.9	5.8
Lys	19	18.5	18.6	17.9	18.6	19.4
At-Lys[c]	0	0.9	0.8	1.2	0.8	1.0
His	12	12.1	11.9	11.9	12.3	11.9
Arg	4	4.0	3.9	3.9	4.1	3.8
Trp[c]	2	2.0	1.9	nd[d]	1.9	1.9
sequence cycle 1:	Val	Leu	Ser	Gly	Glu	Lys
mole fraction:	1.00	1.02	nd	0.97	0.97	0.98
sequence cycle 2:	Leu	Ser	Glu	Leu	Leu	Leu
mole fraction:	1.00	nd	0.96	0.97	0.96	0.95

[a] Protein hydrolysis was performed for 24, 48, and 72 h. Serine and threonine were determined by extrapolation to zero time, while isoleucine, valine, and lysine contents were determined by extrapolation to 100 h. [b] The yield reported under sequence cycle is that fraction of NH_2 terminal possessing the indicated amino acid. [c] Tryptophan and acetimidolysine were determined by the method of Liu & Chang (1971) (Neireiter, 1979). [d] Not determined.

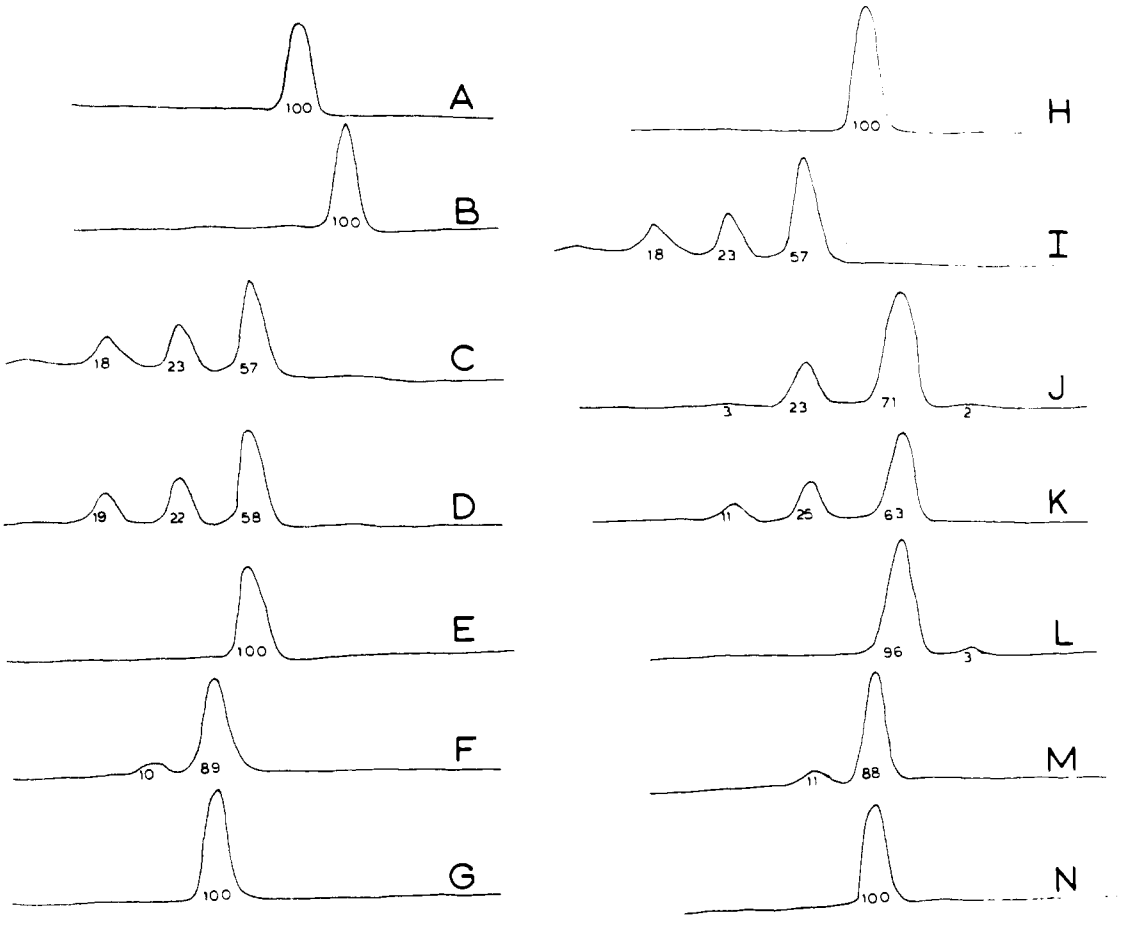

FIGURE 4: Densitometer integration of the electrophoretic separation of the reaction products of maleylation and carbamylation. (A) Untreated myoglobin; (B) acetimidomyoglobin; (C) reaction of maleic anhydride with acetimidomyoglobin shown in (B); (D) reaction of H_4Pht-anhydride with acetimidomyoglobin shown in (B); (E) pure N^{α}-$HO_2CCH{=}CHCO$-$N^{\epsilon}{}_{19}$-acetimidomyoglobin; (F) impure N^{α}-$HO_2CCH{=}CHCO$-myoglobin; (G) N^{α}-$HO_2CCH{=}CHCO$-myoglobin; (H) untreated myoglobin; (I) reaction of maleic anhydride with acetimidomyoglobin shown in (B); (J) carbamylation of acetimidomyoglobin shown in (B); (K) reaction of maleic anhydride with products shown in (J); (L) N^{α}-NH_2CO-$N^{\epsilon}{}_{19}$-acetimidomyoglobin; (M) impure N^{α}-NH_2CO-myoglobin; (N) N^{α}-NH_2CO-myoglobin.

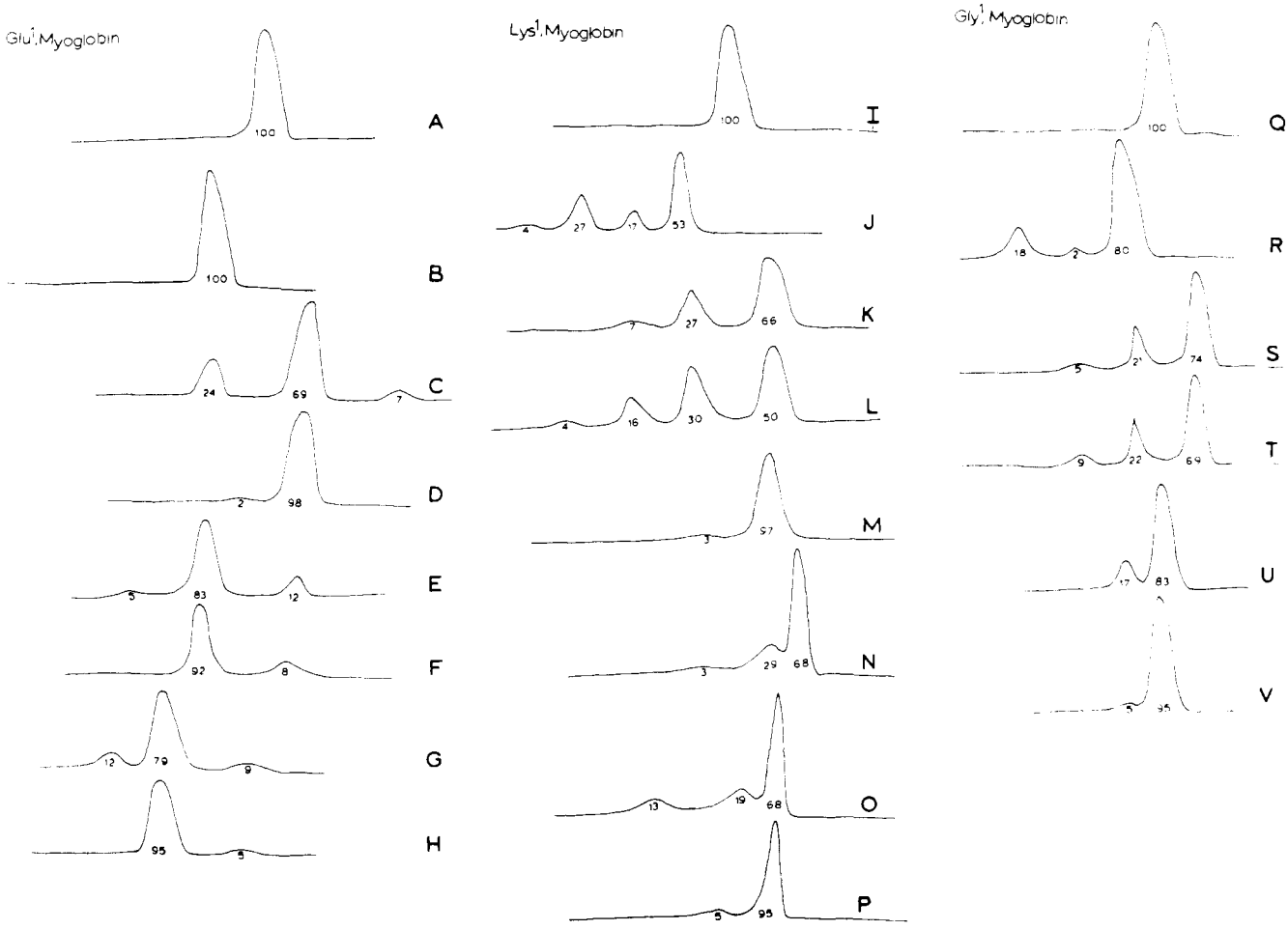

FIGURE 5: Densitometer integration of the electrophoretic separation of the semisynthetic myoglobins and their respective intermediates. (A) Untreated myoglobin; (B) des-Val[1]-N^{α}-H_4Pht-N^{ϵ}_{19}-acetimidomyoglobin; (C) coupling of the ONSu ester of Boc-Glu(OBu^t) to des-Val[1]-N^{ϵ}_{19}-acetimidomyoglobin; (D) N^{α}-Boc-Glu[1](OBu^t)-N^{ϵ}_{19}-acetimidomyoglobin; (E) impure [Glu[1]]-N^{ϵ}_{19}-acetimidomyoglobin; (F) purified [Glu[1]]-N^{ϵ}_{19}-acetimidomyoglobin; (G) impure [Glu[1]]myoglobin following deacetimidation; (H) [Glu[1]]myoglobin; (I) untreated myoglobin; (J) H_4Pht-anhydride reaction with des-Val[1]-acetimidomyoglobin, used in synthesis of [Lys[1]]myoglobin; (K) Boc-Lys(CF_3CO)-ONSu coupling to des-Val[1]-acetimidomyoglobin; (L) H_4Pht-anhydride reaction of products shown in (K); (M) N^{α}-Boc-Lys[1](CF_3CO)-N^{ϵ}_{19}-acetimidomyoglobin; (N) products of trifluoroacetic acid treatment of N^{α}-Boc-Lys[1](CF_3CO)-N^{ϵ}_{19}-acetimidomyoglobin; (O) deacetimidation of products shown in (N); (P) [Lys[1]]myoglobin; (Q) untreated myoglobin; (R) H_4Pht-anhydride reaction with des-Val[1]-acetimidomyoglobin, used in synthesis of [Gly[1]]myoglobin; (S) CF_3CO-Gly-ONSu coupling to des-Val[1]-acetimidomyoglobin; (T) H_4Pht-anhydride reaction of products shown in (S); (U) CM-Sephadex C-50 purified [Gly[1]]myoglobin following deacetimidation; (V) [Gly[1]]myoglobin after additional purification on DEAE-Sephadex A-50.

according to the absence of an NH_2-terminal residue (des-Val[1]- and des-Val[1],Leu[2]), the structure of the first residue side chain ([Glu[1]], [Lys[1]], and [Gly[1]]), and the charge state of the NH_2 terminus (N^{α}-NH_2CO and N^{α}-$HO_2CCH{=}CHCO$).

The primary observation within the first category is the increase (Figure 6, curves 2 and 4) in the net positive charge of the molecule in acid with removal of the first residue[3] and its subsequent return to the native charge with cleavage of the second residue. The des-Val[1]-myoglobin titration is reminiscent of the back-titration of acid-unfolded myoglobin whose increased charge results from the release of the buried histidine residues (Breslow & Gurd, 1962; Hartzell et al., 1968; Friend & Gurd, 1979a). Seven titrating histidine residues in aquoferrimyoglobin (residues 12, 36, 48, 81, 113, 116, and 119) have been assigned by proton nuclear magnetic resonance analysis (Hayes et al., 1975; Botelho, 1975; Botelho & Gurd, 1978). The resonances of three histidine residues (64, 93, and 97) are unobservable by this method due to broadening by the nearby paramagnetic iron atom or constraint within the structure (Wilbur & Allerhand, 1977; Botelho & Gurd, 1978), although His-64 titrates in the pH range below 5.5. The remaining two histidine residues (24 and 82) are internal and do not titrate. They are unaffected by carboxymethylation of the native protein (Hugli & Gurd, 1970a,b; Nigen & Gurd, 1973), and their resonances are broad and remain upfield of the protonated imidazoles (Markley, 1975).

The proton nuclear magnetic resonance spectra of des-Val[1]-myoglobin, N^{α}-NH_2CO-myoglobin, and untreated myoglobin are shown in Figure 7. Clearly, the one resonance corresponding to histidine resonances 48 and 116 (peak 2) in the des-Val[1]-myoglobin has increased its relative area as compared to those of the untreated myoglobin and N^{α}-NH_2CO-myoglobin. This increased area corresponds to one additional protonated histidine residue. In the potentiometric titration of the des-Val[1]-myoglobin (Figure 6, curve 2), the magnitude of the increased positive charge and its location could be accounted for by such an additional titrating imidazole side chain. Apparently, removal of the second residue eliminates the conditions which brought about the titration of this extra histidine residue, as the titration curve was found

[3] This additional net positive charge was observed for des-Val[1]-myoglobin prepared by both synthetic procedures described under Experimental Section.

Table II: Ultraviolet-Visible Absorbance Measurements of the Myoglobin Derivatives

myoglobin derivative	wavelength (nm) absorbance ratio[a] aquoferri 409/280[b]	cyanoferri 423/280	280(CN)/280(H_2O)	hemic acid pKa
native (untreated)	5.33	3.48	1.02	8.88 ± 0.02
N^{α}-NH_2CO	4.41	3.37	1.02	8.67 ± 0.02
N^{α}-HO_2CCH=CHCO	4.94	3.35	1.03	9.01 ± 0.01
[Gly^1]	5.08	nd[c]	nd	nd
[Glu^1]	4.97	3.49	1.03	8.88 ± 0.02
[Lys^1]	5.11	3.65	0.98	8.87 ± 0.02
des-Val^1	5.10	3.49	1.03	8.90 ± 0.02
N^{α}-NH_2CO-des-Val^1	4.38	3.31	1.01	nd
N^{α}-HO_2CCH=CHCO-des-Val^1	4.86	3.27	1.03	nd
des-Val^1,Leu^2	4.95	3.38	1.00	8.91 ± 0.02
N^{α}-NH_2CO-des-Val^1,Leu^2	4.95	3.38	1.00	nd
$N^{\epsilon_{19}}$-At	5.18	3.56	1.02	8.87 ± 0.02
des-Val^1-$N^{\epsilon_{19}}$-At	4.95	3.39	1.00	8.87 ± 0.02
des-Val^1,Leu^2-$N^{\epsilon_{19}}$-At	4.91	3.45	1.02	8.97 ± 0.02

[a] Wavelength absorbance ratios as determined by Nakhleh (1971). [b] Measured at the isoionic point of the protein, except for [Lys^1]-myoglobin which was measured at pH 8.0. [c] Not determined.

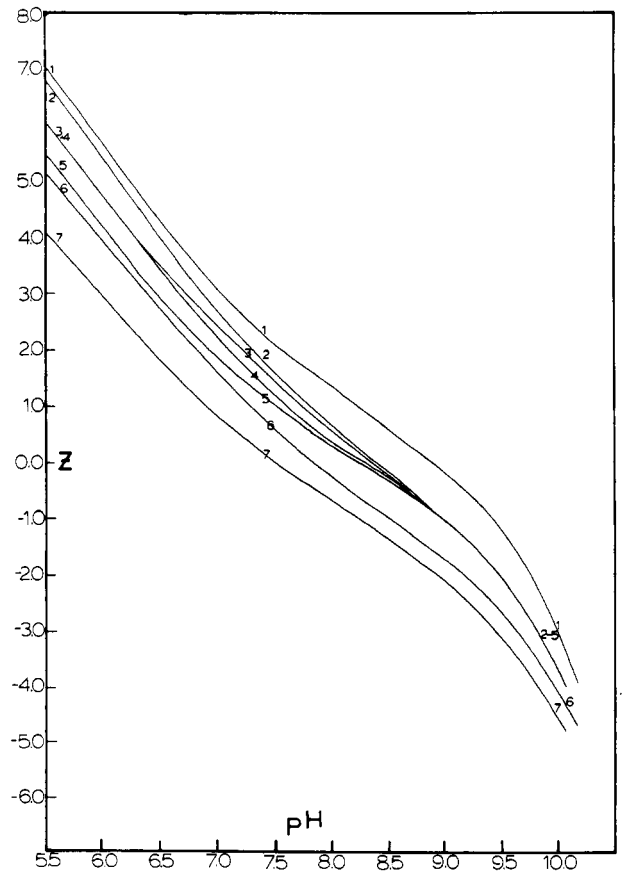

FIGURE 6: Potentiometric hydrogen ion titration curves of the myoglobin derivatives: (1) [Lys^1]myoglobin; (2) des-Val^1-myoglobin; (3) [Gly^1]myoglobin; (4) [Val^1]myoglobin and des-Val^1,Leu^2-myoglobin which show indistinguishable titration curves; (5) N^{α}-NH_2CO-myoglobin; (6) [Glu^1]myoglobin; (7) N^{α}-HO_2CCH=CHCO-myoglobin.

identical with that of native myoglobin (Figure 6, curve 4).

Potentiometric titration of each of the semisynthetic variants showed the disappearance of the excess positive charge noted in the des-Val^1-myoglobin (Figure 6, curves 1, 3, and 6).

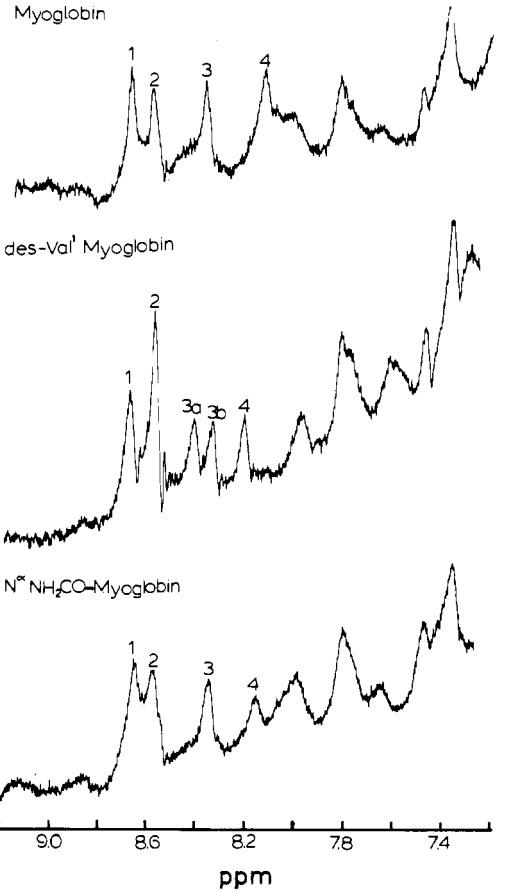

FIGURE 7: Proton C-2 histidine resonances in aquoferrimyoglobin, des-Val^1-myoglobin, and N^{α}-NH_2CO-myoglobin at pH 5.20. Peak 1, histidine residues 12 and 81; peak 2, residues 48 and 116; peak 3, residues 36 and 119; peak 4, residue 113. Assignments are as determined by Botelho & Gurd (1978). No distinction is made as to the assignment of resonances 3a and 3b in the des-Val^1-myoglobin.

Figure 8 shows various differences, ΔZ, between the titration curves of pairs of derivatives, with the cases of blocked NH_2-terminal forms adjusted for comparison in terms of the rest of the molecule. Comparison of the net protein charge of the [Gly^1]myoglobin and native myoglobin revealed a maximum difference of 0.28 charge unit at pH 7.5 (Figure 8E). The magnitude and nature of this charge difference could be accounted for by an increased NH_2-terminal pK for the [Gly^1]myoglobin. This would be in agreement with the findings of Neireiter (1979), who determined the NH_2-terminal pK value of [Gly^1]myoglobin to be 7.59 ± 0.01 and reasoned, after NMR analysis of model pentapeptides, that the Val^1-myoglobin pK should be closer to 7.3. Identification of additional charge interactions in the [Glu^1] and [Lys^1] substitutions (parts C and D of Figure 8) is complicated by the inductive effect of the charged side chains on the α-amino pK (Cohn & Edsall, 1943; Greenstein & Winitz, 1961). However, the large differences in the magnitude and direction between the effects exerted by the [Glu^1] and [Lys^1] substitutions indicate that the remaining polypeptide responds differently to accommodate the variant residues (Steinhardt & Beychok, 1964; Friend & Gurd, 1979b). The pH values at which the maximum differences occur (Figure 8C,D) suggest the perturbation of a side-chain residue with a pK value in the range characteristic of that of a histidine (Botelho & Gurd, 1978).

The N^{α}-NH_2CO substituent attached at the α-amino group of the des-Val^1,Leu^2-myoglobin, the des-Val^1-myoglobin, and the untreated myoglobin yielded nearly identical protein ti-

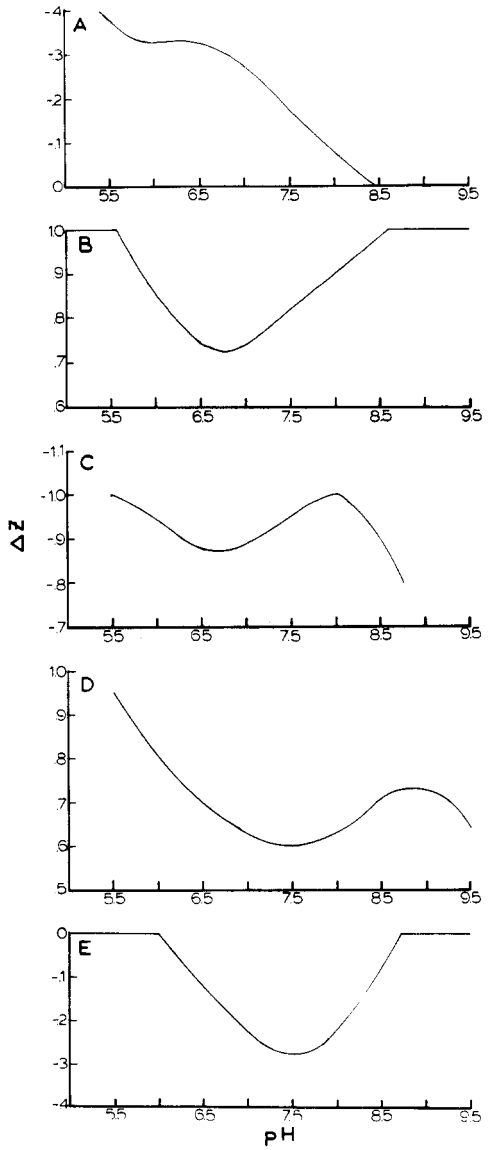

FIGURE 8: Comparison of differences (ΔZ) in the potentiometric titrations of native myoglobin and several of the derivatives prepared. (A) $\Delta Z = Z_{Mb} - (Z_{NH_2CO} + Z_{\alpha\text{-}NH_2})$; (B) $\Delta Z = Z_{Mb} - (Z_{HO_2CCH=CHCO} + Z_{\alpha\text{-}NH_2})$; (C) $\Delta Z = Z_{Mb} - Z_{[Lys^1]}$; (D) $\Delta Z = Z_{Mb} - Z_{[Glu^1]}$; (E) $\Delta Z = Z_{Mb} - Z_{[Gly^1]}$; Z_{Mb} represents the net charge of the native myoglobin, $Z_{\alpha\text{-}NH_2}$ represents the net charge on the NH_2-terminal site assuming a pK value of 7.3, and the other symbols represent the net charges observed with the various derivatized or substituted forms.

tration curves, as was the case with the N^{α}-$HO_2CCH{=}CHCO$ substituent in the latter two proteins (see supplementary material). Analysis of the charge changes imposed on the protein by the NH_2CO and $HO_2CCH{=}CHCO$ substituents is shown in parts A and B of Figure 8. Assuming no changes in the interactions of the NH_2 terminus through carbamylation or maleylation, the expected charge differences would be zero and 1 charge unit, respectively. The N^{α}-$HO_2CCH{=}CHCO$-myoglobin, however, is shown to possess additional net positive charge in the neutral pH range. This could be accounted for by an increase in the pK of the nearby His-81 residue (Takano, 1977) as a result of the new neighboring negative charge (Botelho & Gurd, 1978). In contrast to the mild effects of the maleylated NH_2-terminal residue upon the protein structure, those arising through carbamylation are extensive (Figure 8A) and proceed beyond the local environment, as shown by the lowered hemic acid pK and ultraviolet–visible absorbance spectrum (Table II).

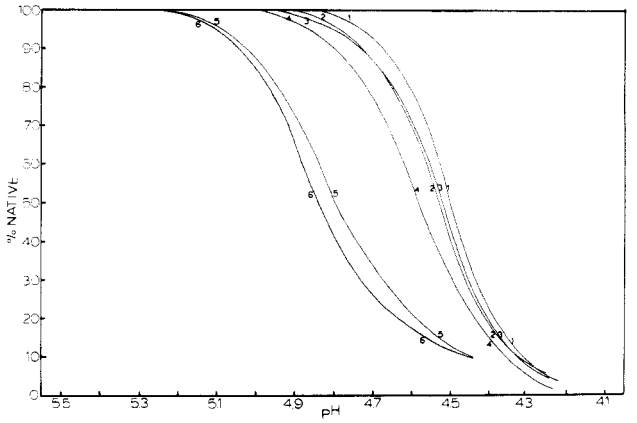

FIGURE 9: Acid stability measurements of myoglobin and NH_2-terminally derivatized myoglobins in 0.1 M citrate at various pH values: (1) myoglobin; (2) N^{α}-NH_2CO-des-Val^1-myoglobin; (3) N^{α}-NH_2CO-myoglobin; (4) des-Val^1-myoglobin; (5) des-Val^1,Leu^2-myoglobin; (6) N^{α}-NH_2CO-des-Val^1,Leu^2-myoglobin.

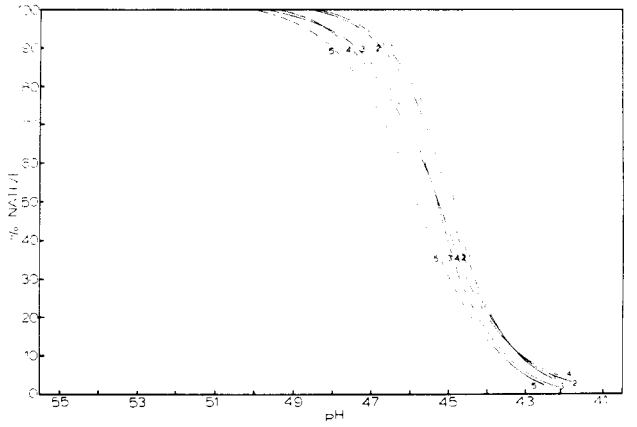

FIGURE 10: Acid stability measurements of myoglobin and three semisynthetic derivatives in 0.1 M citrate at various pH values: (1) [Gly^1]myoglobin; (2) [Val^1]myoglobin; (3) [Lys^1]myoglobin; (4) [Glu^1]myoglobin; (5) des-Val^1-myoglobin.

Acid Stability Measurements. Hartzell et al. (1968) observed that while myoglobins from two different species may have nearly identical titration curves, their stability to acid can differ significantly. Apparently the difference in stability arises from effects of amino acid substitutions on residues intimately involved in the acid unfolding process (Friend & Gurd, 1979a,b; Friend, 1979). Figures 9 and 10 illustrate that almost all of the NH_2-terminally modified myoglobins show differences in their stability to acid as compared to that of native myoglobin. Whereas removal of the first residue increases the midpoint pH of acid denaturation by 0.15 pH unit, cleavage of the second residue increases it an additional 0.30 pH unit (Figure 9). The incorporation of the [Gly^1] residue returns the stability of the des-Val^1-myoglobin to a point nearly identical with that of native myoglobin (Figure 10). In contrast to the [Gly^1] substitution are those with the charged residues, [Glu^1] and [Lys^1], which were found to restore the stability of the molecule only partially but in nearly an equivalent degree (Figure 10). These observations support the hypothesis that the charged side chains of the first residue interact with at least one additional site (Figure 8C,D).

In these stability measurements the N^{α}-NH_2CO derivatives proved quite informative in answering the following question. What fraction of the decreased stability observed for the des-Val^1- and des-Val^1,Leu^2-myoglobins is due to the loss of the first two residues as opposed to that portion which arises

Table III: Circular Dichroism Analysis of Shortened Cyanoferrimyoglobin Derivatives[a]

myoglobin derivative	pH	$[\Theta]_{222}{}^{b} \times 10^{-3}$	$[\Theta]_{274} \times 10^{-1}$	$[\Theta]_{295} \times 10^{-1}$	$[\Theta]_{422} \times 10^{-1}$
myoglobin	6	−21.34	23.86	−9.76	78.67
	10	−21.73	21.77	−9.74	81.35
des-Val[1]-myoglobin	6	−23.97	24.59	−8.34	81.70
	10	−22.86	21.25	−9.46	76.20
des-Val[1],-Leu[2]-myoglobin	6	−24.06	20.05	−14.70	91.57
	10	−23.71	21.44	−14.29	86.41

[a] Determined in $\mu = 0.1$ phosphate buffer at the specified pH in the presence of a 100-fold excess of KCN. [b] $[\Theta]$ is given in units of deg cm^2 dmol^{-1}.

from the new location of the positive charge? The carbamylated products of myoglobin and des-Val[1]-myoglobin showed nearly identical stabilities (Figure 9) which were relatively slightly reduced from those of the parent molecules. Apparently the primary function of the first residue is to maintain the positively charged NH_2 terminus away from the interior of the protein and from several important charge pairs (Friend & Gurd, 1979b). This is in agreement with the finding of only minimal changes in the des-Val[1]-myoglobin at pH values above the pK of the NH_2 terminus (Figure 6 and Tables II and III). The simplest explanation for the small decrease in the stability on carbamylation of the des-Val[1],Leu[2] molecule (Figure 9) is that the removal of the nonpolar side-chain interactions of the invariant second residue is largely responsible for the severely decreased stability of the molecule. In any case, the causes of the altered conformation of the des-Val[1],Leu[2]-myoglobin, as noted by its decreased ability to reassociate with hemin, sharply increased 295-nm molar ellipticity (Table III), increased hemic acid pK (Table II), and decreased acid stability (Figure 9), are not overcome by so small a change as NH_2-terminal carbamylation. Regardless of the interpretation of the carbamylation results, the importance of the second residue is unequivocal.

The results of the stability measurements on the myoglobin derivatives contrasted with those on the acetimidomyoglobin intermediates. Acetimidation of all 19 lysine residues was found to increase the acid denaturation midpoint of the native myoglobin by ~0.25 pH unit. None of the modifications of the NH_2-terminal residue had further effects on the stability relative to the parent $N^{\epsilon}{}_{19}$-acetimidomyoglobin molecule. Removal of the second residue was, as seen in the myoglobin molecule, a destabilizing modification which could not be counteracted through NH_2-terminal carbamylation. Apparently the interactions of the NH_2-terminal residues with the remaining polypeptide have been changed through acetimidation of one or more ϵ-amino groups.

Discussion

Semisynthetic Procedures. Through extension of the quantitative coupling of 3-sulfo-PhNCS to the NH_2 terminus of acetimidoferromyoglobin (Neireiter et al., 1979), a nondestructive method of sequentially removing the NH_2-terminal residues was devised. The $N^{\epsilon}{}_{19}$-acetimidomyoglobin was produced in 58% yield, and prior to purification it was subjected to 3-sulfo-PhNCS coupling, oxidation of the heme to the ferri state, removal of the heme, trifluoroacetic acid cleavage, reconstitution with hemin, and purification to yield the des-Val[1]-acetimidomyoglobin in 38% yield (Figure 1). A 65% yield for the degradation process alone suggested the possibility of sequentially removing several other amino acids. However, repetition of this procedure yielded the des-Val[1],Leu[2]-acetimidomyoglobin in considerably less than the expected yield. Attempts to remove the second residue enzymatically (Light, 1972) were completely unsuccessful.

The presence of the heme throughout the coupling of the 3-sulfo-PhNCS to the acetimidomyoglobin permitted reaction at a pH close to the α-amino-group pK value. Consequently, the conditions found necessary for quantitative coupling were much milder than those employed with the apomyoglobin, leading to a nearly complete avoidance of denaturation in this step (Neireiter et al., 1979). The ability of the acetimidoferromyoglobin to withstand the 3-sulfo-PhNCS coupling conditions was clearly evident after acid cleavage, as reconstitution with hemin could be achieved at high pH (Crumpton & Polson, 1965) in 70% yield with a 409 nm/280 nm absorbance ratio of 4.84 (DiMarchi et al., 1979).

Stability of Truncated and Derivatized Forms. Removal of the first residue proved destabilizing (Figure 9). By comparison with the modest changes in circular dichroism (Table III) and ultraviolet absorbance values (Table II), clear changes are seen in the titration curve (Figure 6), and the appearance of an additional titrating histidine residue (Figure 7) accounts for the increased net positive charge at neutral pH and below. Possible candidates for the newly exposed histidine residue are His-24 and His-82 (Breslow & Gurd, 1962; Botelho & Gurd, 1978; Friend & Gurd, 1979a,b). The facile release of an additional histidine residue could contribute to the decreased stability of the molecule (Breslow & Gurd, 1962; Puett, 1973; Friend & Gurd, 1979a,b). Extensive changes in the tryptophan circular dichroism L_a O–O band at 295 nm have been ascribed to loosening of the anchoring of the A helix at the GH corner (Gurd et al., 1980). It is possible that the exposure of His-82 in the EF region could occur without major disturbance of the anchoring of the A helix but less likely that exposure of His-24 could fail to have a marked effect (Gurd et al., 1980). Further studies should provide positive identification (Hugli & Gurd, 1970a,b; Botelho & Gurd, 1978).

Note that the peculiar titration properties of des-Val[1]-myoglobin might have been ascribed to unexplained damage to the primary structure if it were not that the same preparations were used to rebuild the parent [Val[1]]myoglobin (DiMarchi et al., 1979) and the [Gly[1]]myoglobin reported here.

Removal of the second residue proved decisively more destabilizing than removal of the first. Although the anomaly in titration behavior at neutral pH was removed (Figure 6, curve 4), the des-Val[1],Leu[2]-myoglobin showed lowered stability in acid solution (Figure 9, curve 5), a significantly altered tryptophan ellipticity in particular [Table III; cf. Gurd et al. (1980)], an altered ultraviolet–visible absorption spectrum (Table II), and a reluctance to reassociate properly with hemin. Taken together, these results show substantial alteration of the conformation of the molecule. In the native structure (Takano, 1977) the invariant Leu-2 occupies an internal position so that its removal will require some collapse of the closely packed structure (Gurd & Rothgeb, 1979). It is also positioned in the native structure near the invariant negative charge on the side chain of residue 6, provided in the sperm whale myoglobin by glutamic acid (Friend & Gurd, 1979b). Glu-6 is hydrogen bonded to the amide proton of Ser-3 to stabilize the beginning of the A helix. Glu-6 also forms an invariant charge pair with Lys-133 in the H helix to serve as an anchor for the A and H helices (Friend & Gurd, 1979b). Removal of the Leu-2 residue will disturb such stabilizing interactions both by altering the packing geometry and by

displacing the NH_2-terminal positive charge to reside on Ser-3, a change that can be expected to interfere with the charge pair interaction between Glu-4 and Lys-79 as well (Friend & Gurd, 1979b). The results presented show that the compromise reached in the des-Val[1],Leu[2]-myoglobin preserves the overall titration behavior of the native structure above pH 5.2 but with changes in the tryptophan circular dichroism at 295 nm and some changes in the heme spectra (Tables II and III). The twice-truncated molecule appears to be relatively unstable in the apomyoglobin state and to combine less readily with the heme moiety so that yields of the reconstituted holoprotein were much poorer than with the des-Val[1]-myoglobin.

Carbamylation of the NH_2 terminus was sensed as far away in the molecular structure as the heme, both in terms of absorbance spectrum and in terms of hemic acid pK (Table II). This effect is the more significant because it tallies with the reports by Artyukh et al. (1977, 1979) concerning the methyl isothiocyanate derivative of the α-amino group in sperm whale myoglobin. The structural change in this case makes itself felt in the titration behavior (Figures 6 and 8). The spectral changes indicate a shift in the heme iron spin state toward low spin, in accordance with the observations by Artyukh et al. (1979). Direct electrostatic interaction between the heme iron site and components of the A helix is probably minimal (Friend et al., 1980), so that a conformational effect expressed in other ways is a more likely explanation. This conclusion is supported by the close similarities of most other hemic acid pK values in Table II.

The observation that various NH_2-terminal derivatives of N^{ϵ}_{19}-acetimidomyoglobin differ little in stability among themselves may indicate that the modification of the lysine residues has stabilized the anchoring of the NH_2-terminal region and the A helix to the rest of the molecule. As already pointed out, several charge pairs involving lysine residues play major roles in the interactions of the A helix (Friend & Gurd, 1979b). One or more of these interactions may be strengthened by the conversion to the acetimidolysine form, by either a change in the location of the positive charge, a change in solvent accessibility, an increase in the intrinsic pK value of this residue on conversion to the acetimidolysine, or any combination of these reasons. Although the overall effect of the acetimidation is to reduce the stability of the protein, those interactions localized to the NH_2-terminal region may well undergo a net stabilization.

Effects of Amino Acid Substitution. The close similarity of [Val[1]]- and [Gly[1]]myoglobins, with the exception of a distinct difference in α-amino pK, is defined in greater detail in NMR observations on [Gly[1]]myoglobin enriched in C^{α} of Gly-1 (Neireiter, 1979), in a study that establishes the identity of numerous ^{13}C chemical shift values seen at natural abundance in the two proteins [cf. Wittebort et al. (1979)]. Glycine and valine are the only two amino acids found to occupy the NH_2-terminal position in mammalian myoglobins, with glycine being much more common (Bogardt, 1978). Other planned substitutions of residue 1 may help explain the confinement of this position to glycine and valine.

The substitutions represented in the [Lys[1]]- and [Glu[1]]-myoglobins confer somewhat decreased stability at low pH (Figure 10). The observed changes, however, fall within the range of variation among a group of nine related myoglobins from cetacean species (Friend, 1979). The differences in the titration curves shown in Figure 6 (curves 1 and 6, respectively) reflect the changes in composition, modulated by the compensations in site occupancy (Figure 8), that are to be expected with the introduction of the charge-bearing side chains (Botelho et al., 1978; Friend et al., 1980). The nonadditive titration effects of the Lys-1 and Glu-1 residues mentioned above (Figure 8, curves C and D) presumably represent to some degree particular influences both on the pK of the terminal α-amino group (cf. Figure 8, curve E) and on the pK of His-81.

The effects on the stability of the myoglobin of removing the first residue are striking enough to suggest that the placing of the terminal charge site with respect to the rest of the molecule is important. The first residue is mobile in solution by NMR criteria (Gurd & Rothgeb, 1979; Neireiter, 1979) and in the crystalline state by X-ray diffraction analyses (Frauenfelder et al., 1979). The time-average placement of the terminal α-amino charge site may be required to minimize interactions with the charge pairs Lys-133 with Glu-6 and Lys-79 with Glu-4 as mentioned above, interactions that would be much more threatening if the α-amino charge site were resident on Leu-2.

The results of the present study illustrate the delicate balance which exists between the NH_2-terminal residues and the rest of the protein. The unblocked NH_2 terminus and uncharged side chain in the first residue confer specific properties, and these results will need to be taken into account in interpreting selective pressures in evolution. The advantages of systematic substitution are evident. The present chemical approach involved preliminary truncation of the sequence. Useful as the truncation has proved in the present instance, it is obvious that truncation experiments alone can be misleading as the different truncated forms lead to different structural compromises that may not lend themselves to simple, stepwise interpretations. As will be reported separately (G. W. Neireiter, A. Szabo, and F. R. N. Gurd, unpublished experiments), the preparation of such derivatives as [[2-^{13}C]Gly[1]]myoglobin by the present methods makes possible measurements by NMR that extend considerably the scope of application of these semisynthetic studies.

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Supplementary Material Available

Figures showing trifluoroacetic acid treatment (Figure 11) and potentiometric hydrogen ion titrations (2 pages). Ordering information is given on any current masthead page.

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