or HF molecules, taken as the reactive species, would occur to a lesser extent at high pH (9.2).

Acknowledgments

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Myoglobin Semisynthesis: Removal of the NH_2-Terminal Valine of Sperm Whale Myoglobin and Its Subsequent Reincorporation†

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ABSTRACT: A chemical procedure for selectively removing the NH_2-terminal valine residue of sperm whale myoglobin has been developed. Reaction of N^{ϵ}_{19}-acetimidoapomyoglobin with 3-sulfophenyl isothiocyanate yielded the N^{α}3-sulfoPhNHCS,N^{ϵ}_{19}-acetimidoapomyoglobin. The heme was reintroduced after cleavage of the 3-sulfophenylthiohydantoin of valine in anhydrous trifluoroacetic acid. The des-Val[1],-N^{ϵ}_{19}-acetimidomyoglobin was purified and the homogeneity of the protein was ascertained through electrophoresis before and after reaction with 3,4,5,6-tetrahydrophthalic anhydride, time-course amino acid analysis, and automated stepwise Edman degradation. Investigation of the physical properties of the des-Val[1],N^{ϵ}_{19}-acetimidomyoglobin included near- and far-ultraviolet circular dichroism, potentiometric titration, and ultraviolet–visible absorption measurements. Reconstitution of the sperm whale myoglobin sequence was accomplished through coupling of the *N*-hydroxysuccinimide ester of methylsulfonylethyloxycarbonyl-protected L-valine to the des-Val[1],N^{ϵ}_{19}-acetimidomyoglobin. Removal of the amino-protecting groups yielded a molecule identical with the starting material, contaminated with only a small amount of readily distinguishable byproduct.

Prior to the substitution of any amino acid in a protein, it must be clearly shown that the synthetic procedure employed has in no way altered the structure of the final product. Consequently, all physical changes noted in the final product can be attributed to the substituted residue. In this light, we report an unperturbing chemical procedure for removing the NH_2-terminal valine residue of sperm whale myoglobin and its subsequent covalent reintroduction.

Chemical experimentation directed at replacing any one amino acid in a protein is dependent first on a method of removing it. Ideally the method should be site specific and quantitative while being structurally unperturbing in and of itself. Removal of the NH_2-terminal residue is commonly achieved with phenyl isothiocyanate (Edman, 1950) and its use in preparing semisynthetic proteins is well documented (Offord & DiBello, 1978). Sequence studies have shown this reagent to be highly quantitative with nearly all peptides (Edman & Henschen, 1975). However, in protein semisynthesis its inability to couple selectively with the α-amino group in the presence of several ϵ-amino groups is a severe disadvantage. Furthermore, its extremely low water solubility and the necessary presence of a strong anhydrous acidic solvent to effect cleavage are two additional drawbacks. Three milder methods of removing the NH_2-terminal valine of sperm whale myoglobin have been explored (Garner, 1974). Cleavage with aminopeptidase M (Wachsmuth et al., 1966) or (triethylenetetramine)cobalt(III) ion (Bentley & Creaser, 1973) or through transamination (Dixon & Fields, 1972) was found not as efficient or reliable as the Edman procedure.

As a consequence of the irreversible denaturation of myoglobin in nonaqueous media (Herskovitz & Solli, 1975; Herskovitz et al., 1977), a hydrophilic Edman reagent, 3-sulfophenyl isothiocyanate (Dwulet & Gurd, 1976) was used in the present work. Selective coupling to the α-amino group of the apoprotein was directed through acetimidyl protection of the ϵ-amino groups (DiMarchi et al., 1978b). The 3-sulfophenylthiohydantoin formed simplifies the subsequent purifications following coupling and cleavage by virture of its

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additional negative charge (DiMarchi et al., 1978a).

Cleavage of the 3-sulfophenylthiohydantoin of valine was achieved in anhydrous trifluoroacetic acid after which the heme was reintroduced and the des-Val^1,N^{ϵ}_{19}-acetimidomyoglobin[1] was purified. The removal of the NH_2-terminal residue increased the net charge of the molecule in the range between pH 6.5 and 9.5. This change had little effect on the coupling of Msc-valine-ONSu to the newly formed α-amino group, as it was found only slightly less efficient than the coupling to the original NH_2-terminal valine (DiMarchi, 1978). Comparison of the potentiometric titrations of the N^{ϵ}_{19}-acetimidomyoglobin and the N^{α}Msc,N^{ϵ}_{19}-acetimidomyoglobin yielded an apparent pK of 7.20 ± 0.10 for the NH_2-terminal valine. Removal of all the amino-protecting groups yielded a myoglobin which was identical with the parent material as suggested by characterization by several physical methods.

Materials and Methods

The isolation and purification of the principal and minor components of sperm whale myoglobin were carried out as previously described (Hapner et al., 1968). The principal component IV was used as the starting material for this synthetic study. All reagents and buffers (pico buffer system II) for the amino acid analyzer were purchased from Pierce as were the *N*-hydroxysuccinimide, trifluoroacetic acid (Sequanal grade), 3-sulfo-PhNCS, methylthiohydantoin of various amino acids, and dicyclohexylcarbodiimide. The reagents for the amino acid sequence analysis were sequencer grade reagents from Beckman. The 2-methylmercaptoethanol was purchased from Pfaltz and Bauer. Bovine hemin chloride was obtained from Sigma. The urea was deionized before use by passing through a column of Rexyn I-300 (Fisher), and all other chemicals were reagent grade quality.

The experimental methods used in amino acid analysis, NH_2-terminal sequence determination, potentiometric hydrogen ion titrations, electrophoresis, and reaction of 3,4,-5,6-tetrahydrophthalic anhydride with acetimidomyoglobin are described in a previous paper (DiMarchi et al., 1978b). The modification and isolation of a myoglobin derivative bearing acetimidyl protection at all 19 lysine residues but retaining a free amino group has been reported (DiMarchi et al., 1978b).

Ultraviolet and Visible Absorption Measurements. Spectra of the aquoferrimyoglobins were measured at each wavelength, for three different concentrations, with a Zeiss PMQII spectrophotometer. After analysis at each concentration, the protein was converted to the low-spin cyanoferrimyoglobin derivative and the absorbance maxima were noted once more. The absorbance readings at each wavelength were fitted to a Beer's law relationship, and the slope of each line was determined. Comparison of the slopes at different wavelengths provides ratios of the extinction coefficients. The semisynthetic myoglobin was reduced to the ferro derivative with sodium dithionite and converted to the carboxy form under a steady stream of carbon monoxide. Its visible absorbance spectrum was recorded with a Cary Model 14 spectrophotometer and compared with that of the untreated carboxyferromyoglobin (Hardman et al., 1966).

Circular Dichroism Measurements. The circular dichroism spectra were recorded at 25 °C under nitrogen on a Jasco optical rotatory dispersion recorder with a Sproul Scientific SS-10 CD modification which allowed circular dichroism measurements to be made. Calibration of the instrument was done with *d*-camphor-10-sulfonic acid. The amount of α-helical secondary structure was calculated by the method of Chen et al. (1972).

Synthesis of Msc-L-valine-ONSu. The Msc-L-valine was synthesized following the procedure of Tesser & Balvert-Geers (1975) with an overall yield of 20% starting from 2-methylmercaptoethanol. Recrystallization of the Msc-L-valine from methanol–diethyl ether yielded white crystals melting at 125–126 °C with an $[\alpha]^{20}_D = -0.07°$ (1% in methanol). To 1.5 mmol (400 mg) of the Msc-L-valine dissolved in a minimum amount of acetonitrile was added an equivalent amount (172 mg) of *N*-hydroxysuccinimide. Upon cooling the solution to 0 °C, 1.5 mmol (309 mg) of dicyclohexylcarbodiimide was added with stirring. After 24 h, the dicyclohexylurea was filtered and the solvent was removed under vacuum. The oil which remained was solubilized in warm acetic acid and the Msc-L-valine-ONSu was crystallized with the addition of diisopropyl ether. Thin-layer chromatography in 60:30:1:4 (chloroform:methanol:water:acetic acid) revealed only one spot with an R_f value of 0.83: yield, 63%; mp 134–135 °C; and $[\alpha]^{24}_D = +38.8°$ (0.5% in methanol).

Reaction of 3-Sulfophenyl Isothiocyanate with N^{ϵ}_{19}-Acetimidoapomyoglobin. The heme was removed from the N^{ϵ}_{19}-acetimidoferrimyoglobin (DiMarchi et al., 1978b) with 2-butanone at pH 3.8 in the presence of 0.1 M sodium fluoride (Yonetani, 1967), dialyzed against water, and freeze-dried. The lyophilized N^{ϵ}_{19}-acetimidoapomyoglobin was dissolved to 10 mg/mL in a deoxygenated 5 M urea solution equilibrated at 35 °C. Double coupling to the protein at pH 6.0 was achieved through two 20-fold excess additions of 3-sulfo-PhNCS at a 2-h interval. After 4 h, the protein solution was adjusted to be 8 M in urea and was removed from excess 3-sulfo-PhNCS by passage through a Rexyn I-300 column in 8 M urea. The urea was removed through dialysis against deionized water and the protein was freeze-dried.

Cleavage of the NH_2-Terminal Residue. To 1.0 g of the lyophilized N^{α}3-sulfo-PhNHCS,N^{ϵ}_{19}-acetimidoapomyoglobin were added 200 mg of dithioerythritol and 100 μL of anisole with 10 g of Sequanal grade anhydrous trifluoroacetic acid. The protein was swirled in a nitrogen environment for 30 min following dissolution. Flash evaporation under water-aspirated vacuum in a nitrogen atmosphere removed the majority of the acid, while any residual amounts were removed under an oil-pumped vacuum. The cleaved protein was rehydrated in deionized water to a final concentration of 3 mg/mL and subsequently dialyzed against water at 4 °C until a pH of approximately 6.0 was obtained.

Reconstitution of the Des-Val^1,N^{ϵ}_{19}-acetimidoapomyoglobin with Hemin. To a 1% solution of the des-Val^1,N^{ϵ}_{19}-acetimidoapomyoglobin, as determined by amino acid analysis, adjusted to pH 4.5 with glacial acetic acid was added a stoichiometric amount of bovine hemin chloride in a minimum amount of absolute pyridine. The pH was maintained between 4.7 and 4.8 with the addition of glacial acetic acid as the reconstitution proceeded for 30 min at 4 °C. Excess reagents were removed by dialysis against deionized water over a 24-h period. Any hemochromogen formed was then precipitated through dialysis against 0.1 μ, pH 6.5 phosphate buffer at 4 °C and removed by centrifugation at 5000 rpm. The des-Val^1,N^{ϵ}_{19}-acetimidomyoglobin was isolated on CM-Sephadex

[1] Abbreviations used: A_2C_2, acetimidomyoglobin bearing a free NH_2 terminus and nearly complete ϵ-amino modification (DiMarchi et al., 1978b); At, acetimidyl; des-Val^1-, myoglobin which lacks the NH_2-terminal residue; Msc, methylsulfonylethyloxycarbonyl; ONSu, *N*-hydroxysuccinimide ester; PhNHCS, phenylthiocarbamoyl; 3-sulfo-PhNCS, 3-sulfophenyl isothiocyanate; 3-sulfo-PhNHCS, 3-sulfophenylthiocarbamoyl; H_4Pht, 3,4,5,6-tetrahydrophthalyl; N^{α}, modification at the NH_2-terminal nitrogen atom; N^{ϵ}_{19}, modification at the ϵ-amino nitrogen atom of all 19 lysine residues.

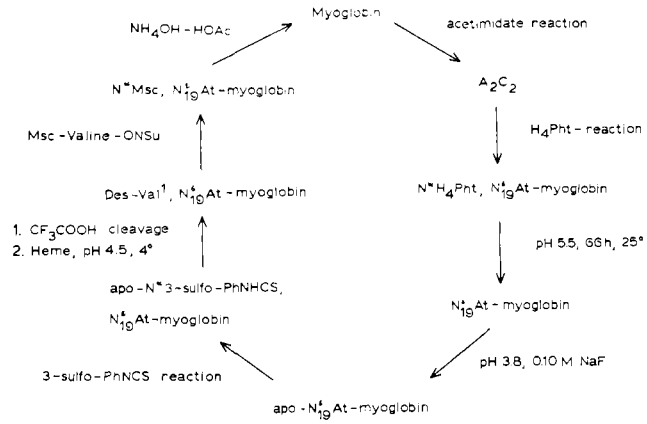

FIGURE 1: Flow chart outlining the synthetic procedure for removal of NH_2-terminal valine and its subsequent replacement. All abbreviations are defined in footnote 1.

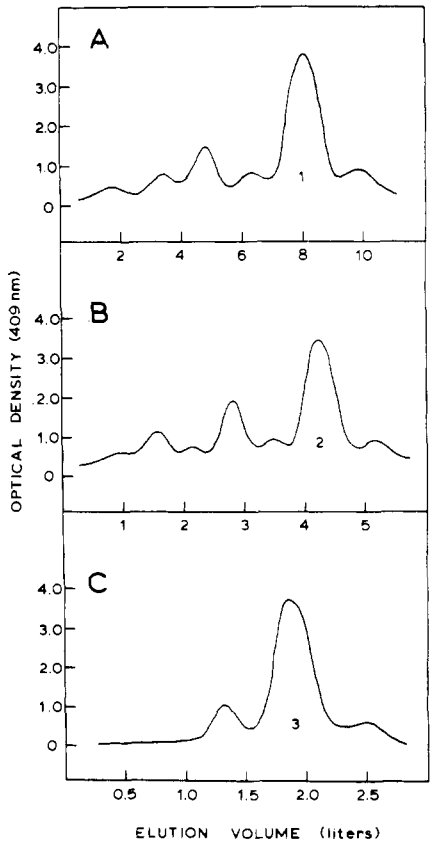

FIGURE 2: The elution profiles of chemically modified myoglobins. All purifications utilized CM-Sephadex C-50 resin with 0.1 μ phosphate as the eluting buffer. (A) Products of acetimidation reaction on a column 9.5 × 50 cm at pH 6.50, 25 °C, 750 mL/h. (B) Products after reaction of acetimidomyoglobin with 3,4,5,6-tetrahydrophthalic anhydride on a column 9.5 × 50 cm at pH 6.40, 4 °C, 350 mL/h. (C) Products after removal of the 3,4,5,6-tetrahydrophthalyl group on a column 5.0 × 50 cm at pH 6.50, 25 °C, 350 mL/h. The number under each major fraction refers to (1) A_2C_2;[1] (2) $N^{\alpha}H_4$Pht,N^{ϵ}_{19}-acetimidomyoglobin; (3) N^{ϵ}_{19}-acetimidomyoglobin.

C-50, eluting with phosphate buffer, pH 6.45, μ = 0.1, 25 °C.

Reaction of Msc-L-valine-ONSu with Des-Val^1,N^{ϵ}_{19}*-acetimidomyoglobin.* To a 1% solution of des-Val^1,N^{ϵ}_{19}-acetimidomyoglobin was added a 20-fold excess of Msc-L-valine-ONSu. The reaction was allowed to proceed for 3 h at pH 8.0, 15 °C. Excess reagents were removed from the protein by dialysis before purification on CM-Sephadex C-50. Concomitant removal of the two types of amino-protecting groups was achieved by exposure of the ferrimyoglobin derivative to concentrated ammonium hydroxide–acetic acid (15:1, v/v) at an apparent pH 11.5, 16 °C for 28 h (Ludwig & Byrne, 1962; Garner & Gurd, 1975).

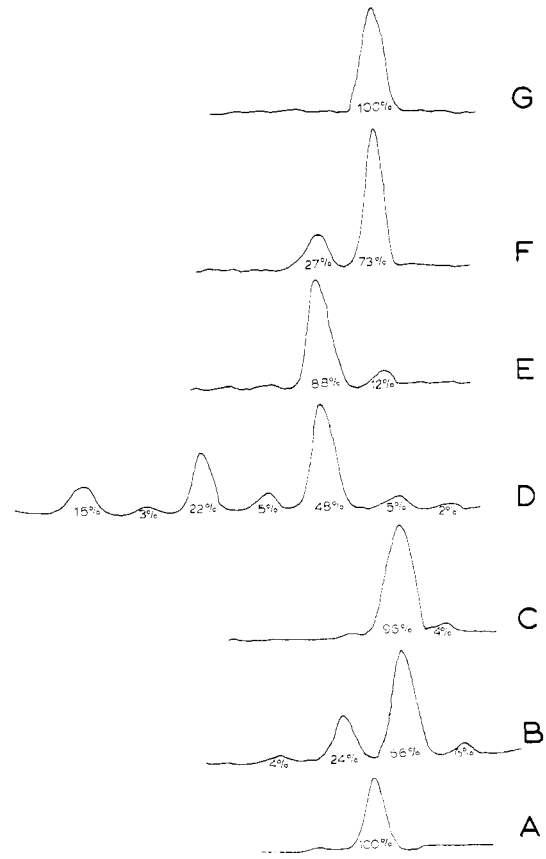

FIGURE 3: Densitometer integration of the electrophoretic separation of the untreated myoglobin and acetimidomyoglobin derivatives at pH 9.2, 300 V, in 0.1 M Tris–EDTA–boric acid. (A) Myoglobin; (B) acetimidation of myoglobin; (C) A_2C_2;[1] (D) 3,4,5,6-tetrahydrophthalylation of A_2C_2;[1] (E) $N^{\alpha}H_4$Pht,N^{ϵ}_{19}-acetimidomyoglobin;[3] (F) removal of $N^{\alpha}H_4$Pht group; (G) N^{ϵ}_{19}-acetimidomyoglobin.

Results

Figure 1 illustrates the synthetic procedure for removal of the NH_2-terminal valine residue and its subsequent covalent reintroduction.

Isolation of N^{α}3-Sulfo-PhNHCS,N^{ϵ}_{19}-acetimidoapomyoglobin. Reaction of a sixfold excess of methyl acetimidate with a 3% solution of sperm whale myoglobin was conducted at pH 10.7 ± 0.1, 15 °C, for 30 min in an effort to maximize the production of N^{ϵ}_{19}-acetimidomyoglobin (DiMarchi et al., 1978b). Separation of the acetimidomyoglobin derivatives at pH 6.5 on CM-Sephadex C-50 (Figure 2A) in 0.1 μ phosphate buffer yielded the desired fraction in 64% yield (Table I). Acetimidomyoglobin derivatives bearing less than complete ϵ-amino modification were removed on CM-Sephadex C-50 at pH 6.4, 4 °C, after reaction with 3,4,5,6-tetrahydrophthalic anhydride (Figure 2B). The $N^{\alpha}H_4$Pht,N^{ϵ}_{19}-acetimidomyoglobin can be easily recognized by electrophoresis at pH 9.2 since it possesses one additional negative charge relative to the N^{ϵ}_{19}-acetimidomyoglobin (Figure 3E,G). Removal of the 3,4,5,6-tetrahydrophthalyl group from the α-amino group was found more difficult than from the ϵ-amino group (DiMarchi et al., 1978b). It took 66 h of dialysis at 25 °C against 0.1 μ phosphate buffer, pH 5.5, to remove 73%, as compared with 30 h at pH 6.0 to displace a comparable proportion from lysine-77. Purification of the N^{ϵ}_{19}-acetimidomyoglobin from the $N^{\alpha}H_4$Pht,N^{ϵ}_{19}-acetimidomyoglobin was achieved at pH 6.5

Table I: Amino Acid Composition,[a] NH_2-Terminal Analysis,[b] and Yields[c] of the Acetimidated Derivatives

amino acid	myoglobin	A_2C_2	$N^{\alpha}H_4$Pht,$N^{\epsilon}{}_{19}$-acetimido-myoglobin	$N^{\epsilon}{}_{19}$-acetimido-myoglobin	N^{α}3-sulfo-PhNHCS,$N^{\epsilon}{}_{19}$-acetimidoapo-myoglobin	des-Val[1],-$N^{\epsilon}{}_{19}$-acetimido-myoglobin	N^{α}Msc,$N^{\epsilon}{}_{19}$-acetimido-myoglobin	semisynth myoglobin
Asp	8	8.7	7.7	7.9	8.2	8.6	8.5	8.3
Thr	5	5.1	4.8	4.9	4.9	5.2	4.7	5.0
Ser	6	5.9	6.1	6.0	6.3	6.0	5.7	5.7
Glu	19	19.4	19.0	19.0	19.3	18.9	19.3	19.5
Pro	4	4.1	4.0	3.8	3.9	4.0	4.0	4.1
Gly	11	11.1	10.9	10.8	11.2	11.2	11.4	11.2
Ala	17	17.8	17.7	17.7	16.7	17.3	17.7	17.7
Val	8	8.0	8.2	7.9	7.7	7.0	8.0	7.9
Met	2	2.0	1.8	1.9	2.2	2.0	1.9	2.1
Ile	9	9.4	8.4	8.6	9.1	8.9	8.4	9.4
Leu	18	17.5	18.4	18.2	18.2	17.6	18.9	18.6
Tyr	3	2.6	2.8	3.1	3.0	3.0	3.0	3.1
Phe	6	5.5	5.9	6.1	6.3	6.1	6.4	6.4
Lys	19							18.5
His	12	12.5	12.4	12.2	12.8	12.0	12.5	11.6
Arg	4	3.5	4.0	3.9	4.0	3.9	3.7	3.7
Trp[d]	2	nd[e]	nd	2.0	nd	2.0	nd	nd
acetimido-Lys		18.4	18.7	19.1	19.9	18.8	19.1	
sequencer cycle								
round 1 Val	1.00	1.04	0.00	1.01	<0.04	0.02	0.07	>0.95
round 1 Leu	0.00	0.00	0.00	0.00	0.00	0.98	0.00	0.00
round 2 Val	0.00	0.00	0.58	0.00	>0.96		0.00	0.00
round 2 Leu	1.00	0.94	0.00	0.97	0.04		0.05	>0.95
% yields	100	64	30	20	19	1.2	0.50	0.31

[a] Protein hydrolysis was performed for 24, 48, and 72 h. Serine, threonine, lysine, and acetimidolysine content were determined by extrapolation to zero time while isoleucine and valine contents were determined by extrapolation to 100 h. A_2C_2 is defined in the first footnote. [b] The yield reported under sequencer cycle is that fraction of amino acid found at the NH_2 terminus. [c] The yield reported is for the amount isolated after purification. [d] Tryptophan was determined by the method of Liu & Chang (1971). [e] nd represents not determined.

(Figure 2C). The homogeneity of the product was confirmed by electrophoresis (Figure 3F,G).

Removal of the heme prior to the 3-sulfo-PhNCS coupling avoided the binding of the isothiocyanate to the heme, heme-catalyzed oxidation of the resultant thiourea, or subsequent hemin precipitation upon trifluoroacetic acid cleavage. A single 20-fold excess of 3-sulfo-PhNCS resulted in modification of 92% of the NH_2 terminus, while exposure to an additional 20-fold excess increased the yield to 96%. Urea in a concentration of 5 M was necessary to maintain the protein in a water-soluble state throughout the course of the reaction.

Cleavage of 3-Sulfo-PhNHCS-valine. A chemical procedure was devised that minimized any destruction of the protein upon exposure to trifluoroacetic acid. All amino acids with a remote possibility of partial destruction were first tested by treatment with trifluoroacetic acid. The quantity of amino acid was determined by amino acid analysis before and after treatment.[2] Only methionine and tryptophan were affected significantly and their integrity was found to be preserved through the addition of 20 mg of dithioerythritol and 10 μL of anisole to each milliliter of trifluoroacetic acid.

Before attempting to cleave the NH_2-terminal residue under these conditions, the effects upon the protein were assessed through similar treatment of the $N^{\epsilon}{}_{19}$-acetimidoapomyoglobin. The 280 nm/250 nm absorbance ratio was monitored closely (Figure 4), since it is known to be sensitive to changes in the aromatic side chains (Savige & Fontana, 1978). Trifluoroacetic acid treatment of the apoprotein altered its ultraviolet spectrum only slightly, and tryptophan analysis before and after treatment yielded 1.97 and 1.96 residues per molecule, respectively. The heme was reintroduced to the trifluoroacetic acid treated apoprotein to yield a 409 nm/280 nm absorbance ratio of 4.73, while electrophoresis showed 83% of the protein moving isoelectrically at pH 9.2 with $N^{\epsilon}{}_{19}$-acetimidomyoglobin (Figure 5B,C).

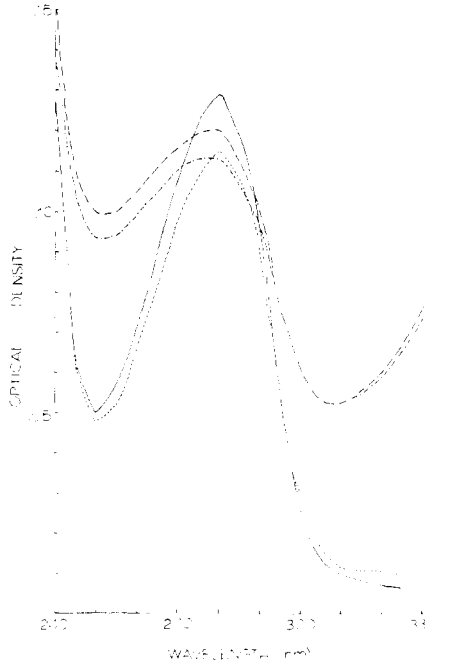

FIGURE 4: Ultraviolet absorbance spectrum of $N^{\epsilon}{}_{19}$-acetimidomyoglobin (-----), $N^{\epsilon}{}_{19}$-acetimidoapomyoglobin (—), trifluoroacetic acid treated $N^{\epsilon}{}_{19}$-acetimidoapomyoglobin (---), rehemed trifluoroacetic acid treated $N^{\epsilon}{}_{19}$-acetimidomyoglobin (– – –).

As a consequence of these encouraging results, the trifluoroacetic acid treatment was extended to the N^{α}3-sulfo-PhNHCS,$N^{\epsilon}{}_{19}$-acetimidoapomyoglobin. Removal of 73% of the 3-sulfo-PhNHCS-valine was noted. Oxidative desul-

[2] Experimental data are presented in the supplementary material (see paragraph at end of paper).

[3] The smaller band results from partial removal of the $N^{\alpha}H_4$Pht– with time.

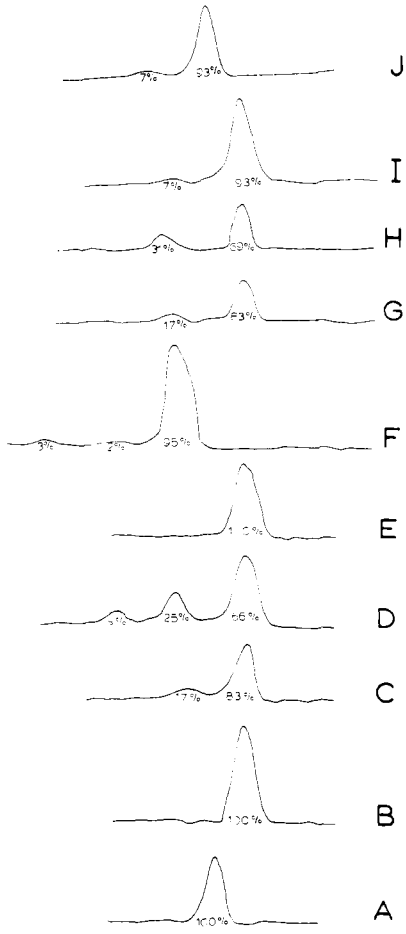

FIGURE 5: Densitometer integration of the electrophoretic separation of myoglobin and the acetimidomyoglobin derivatives at pH 9.2, 300 V, in 0.1 M Tris–EDTA–boric acid. (A) Myoglobin; (B) N^{ϵ}_{19}-acetimidomyoglobin; (C) rehemed trifluoroacetic acid treated N^{ϵ}_{19}-acetimidomyoglobin; (D) impure des-Val^1,N^{ϵ}_{19}-acetimidomyoglobin; (E) des-Val^1,N^{ϵ}_{19}-acetimidomyoglobin; (F) 3,4,5,6-tetrahydrophthalylation of des-Val^1,N^{ϵ}_{19}-acetimidomyoglobin; (G) coupling products of Msc-valine-ONSu to des-Val^1,N^{ϵ}_{19}-acetimidomyoglobin; (H) 3,4,5,6-tetrahydrophthalylation of impure N^{α}Msc,N^{ϵ}_{19}-acetimidomyoglobin; (I) N^{α}Msc,N^{ϵ}_{19}-acetimidomyoglobin; (J) semisynthetic myoglobin.

furization was presumably responsible for the failure of the remaining protein to cleave (Edman & Henschen, 1975).

Characterization of N^{ϵ}_{19}-Acetimidoapomyoglobin. Isoionic point determinations of the N^{ϵ}_{19}-acetimidoapomyoglobin, N^{α}3-sulfo-PhNHCS,N^{ϵ}_{19}-acetimidoapomyoglobin, and trifluoroacetic acid treated N^{α}3-sulfo-PhNHCS,N^{ϵ}_{19}-acetimidoapomyoglobin preparations at 100 μM concentration in 8 M urea yielded pI values of 10.04, 8.05, and 9.18, respectively. On the basis of the wide variation in the isoelectric points of these derivatives, an attempt was made to purify the des-Val^1,N^{ϵ}_{19}-acetimidoapomyoglobin by ion-exchange chromatography. Purification on DEAE-Sephadex A-50 in a 25 mM sodium acetate–25 mM sodium barbital–13 mM EDTA/8 M urea buffer was complicated by aggregation of the apoprotein.[2] The formation of aggregated hybrids when more than one acetimidoapomyoglobin derivative was present made purification of the trifluoroacetic acid treated N^{α}3-sulfo-PhNHCS,N^{ϵ}_{19}-acetimidoapomyoglobin impractical.

Purification of Holo-des-Val^1,N^{ϵ}_{19}-acetimidomyoglobin. Reintroduction of the heme prior to isolation of the des-Val^1,N^{ϵ}_{19}-acetimidoapomyoglobin was complicated by the presence of byproducts of the coupling and cleavage steps. Table II shows the yield of protein obtained through reheming

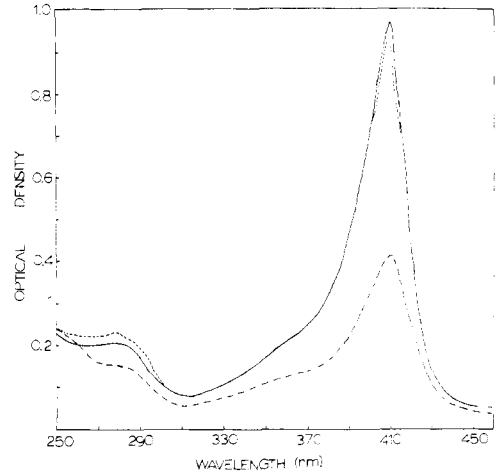

FIGURE 6: Ultraviolet–visible absorbance spectrum of the aquoferri-des-Val^1-acetimidomyoglobin derivatives. Impure des-Val^1,-N^{ϵ}_{19}-acetimidomyoglobin before precipitation of hemochromogen (---); impure des-Val^1,N^{ϵ}_{19}-acetimidomyoglobin after precipitation of hemochromogen (---); des-Val^1,N^{ϵ}_{19}-acetimidomyoglobin after purification on CM-Sephadex C-50 (—) (Figure 7).

under various conditions. An extremely low yield of holoprotein, which furthermore possessed a significantly reduced 409 nm/280 nm absorbance ratio, was obtained through reheming of the unpurified apoprotein. It was apparent that the 409 nm/280 nm absorbance ratio had been diminished much more than could be accounted for by contamination with the uncleaved N^{α}3-sulfo-PHNHCS,N^{ϵ}_{19}-acetimidoapomyoglobin.

The possibility that excess 3-sulfo-PhNCS or the cleaved 3-sulfophenylthiohydantoin of valine remained bound noncovalently to the apoprotein following the cleavage step was substantiated after passage of the product of the trifluoroacetic acid treatment of N^{α}3-sulfo-PHNHCS,N^{ϵ}_{19}-acetimidoapomyoglobin through a Sephadex G-10 column in 6 M urea/0.1 M acetate.[2] All of the protein applied to the gel filtration column was recovered free of any contaminating salts or small molecule byproducts. Reheming of the apoprotein, following extensive dialysis against water to remove urea, showed a slight decrease in the yield of holoprotein yet with an increased 409 nm/280 nm absorbance ratio as compared with that obtained on reheming of the unpurified apoprotein.

The denaturing effect of the contaminating noncovalently bound material was investigated through passage of the product of the trifluoroacetic acid treatment of N^{α}3-sulfo-PHNHCS,N^{ϵ}_{19}-acetimidoapomyoglobin through a Rexyn I-300 mixed bed ionic exchange column in urea. While a decrease of nearly fourfold in the 280-nm absorbance was detected for the trifluoroacetic acid treated N^{α}3-sulfo-PHNHCS,N^{ϵ}_{19}-acetimidoapomyoglobin,[2] no change was noted in the unmodified N^{ϵ}_{19}-acetimidoapomyoglobin after subjection to the same conditions. Passage of the former in 8 M urea as opposed to 5 M urea yielded an additional 16% decrease in the 280-nm absorbance. Amino acid analysis before and after column purification revealed that nearly 50% of the trifluoroacetic acid treated N^{α}3-sulfo-PhNHCS,N^{ϵ}_{19}-acetimidoapomyoglobin had bound to the column. Therefore, the high initial absorbance at 280 nm resulted not only from noncovalently bound salts but also presumably from aggregated apoprotein.

Reintroduction of the heme to the Rexyn-purified, trifluoroacetic acid treated apomyoglobin was found most successful at low pH by using pyridine as the heme solvent. The 409 nm/280 nm absorbance ratio after precipitation of

Table II: Reintroduction of Heme to the Acetimidoapomyoglobins

acetimidyl derivative	heme solvent	temp (°C)	time (min)	pH	purifien of apoprotein[a]	409/280[b]	% yield
CF_3COOH-treated N^{α}3-sulfo-PhNHCS,$N^{\epsilon}{}_{19}$-acetimidomyoglobin	NaOH (0.05 M)	2	30	10.5	none	0.35	4
myoglobin	NaOH (0.05 M)	2	30	10.5	none	5.02	95
$N^{\epsilon}{}_{19}$-acetimidomyoglobin	NaOH (0.05 M)	2	30	10.5	none	4.73	92
CF_3COOH-treated N^{α}3-sulfo-PhNHCS,$N^{\epsilon}{}_{19}$-acetimidomyoglobin	NaOH (0.05 M)	2	30	4.5	none	0.59	13
CF_3COOH-treated N^{α}3-sulfo-PhNHCS,$N^{\epsilon}{}_{19}$-acetimidomyoglobin	py[d]	2	30	4.5	none	0.97	23
myoglobin	py	2	30	4.5	none	4.26	7
$N^{\epsilon}{}_{19}$-acetimidomyoglobin	py	2	30	4.5	none	4.14	52
CF_3COOH-treated N^{α}3-sulfo-PhNHCS,$N^{\epsilon}{}_{19}$-acetimidomyoglobin	py	5	30	4.5	Rexyn I-300	4.02	46
CF_3COOH-treated N^{α}3-sulfo-PhNHCS,$N^{\epsilon}{}_{19}$-acetimidomyoglobin	py	25	30	4.5	Rexyn I-300	2.80	nd[c]
CF_3COOH-treated N^{α}3-sulfo-PhNHCS,$N^{\epsilon}{}_{19}$-acetimidomyoglobin	py	35	30	4.5	Rexyn I-300	2.50	nd
CF_3COOH-treated N^{α}3-sulfo-PhNHCS,$N^{\epsilon}{}_{19}$-acetimidomyoglobin	py	5	60	4.5	Rexyn I-30	4.00	nd
CF_3COOH-treated N^{α}3-sulfo-PhNHCS,$N^{\epsilon}{}_{19}$-acetimidomyoglobin	py	5	30	4.5	Sephadex G-10	2.32	18

[a] The method employed to purify the apomyoglobin prior to reheming. The Rexyn I-300 and Sephadex G-10 columns were run in 8 M urea and 6 M urea/0.1 M acetate, pH 5.0, respectively. [b] The 409/280 absorbance ratio observed following precipitation of any hemochromogen. [c] nd represents not determined. [d] py, pyridine.

most of the hemochromogen increased from 2.70 to 4.03 (Table II, Figure 6). The des-Val[1],$N^{\epsilon}{}_{19}$-acetimidomyoglobin was then purified on CM-Sephadex C-50 at pH 6.45 in 0.1 μ phosphate (Figure 7A). It was identified by amino acid analysis and NH_2-terminal sequence determination[2] (Table I). The possibility of partial removal of the acetimidyl groups during trifluoroacetic acid treatment was eliminated through electrophoretic analysis following coupling with 3,4,5,6-tetrahydrophthalic anhydride (Figure 5E,F). Only one component was seen moving with a net charge of −1 relative to the uncoupled material, characteristic of addition of the 3,4,5,6-tetrahydrophthalyl group at the NH_2 terminus.

Re-formation of the Myoglobin Primary Structure. The L-valine residue was reintroduced as the methylsulfonylethyloxycarbonyl, amino-protected *N*-hydroxysuccinimide ester. This amino-protecting group aids water solubility, minimizes racemization, and is removable under the conditions of deacetimidation (Tesser & Balvert-Geers, 1975). A coupling yield of 86% (Figure 5G,H) to the NH_2-terminal leucine of des-Val[1],$N^{\epsilon}{}_{19}$-acetimidomyoglobin was nearly 90% of that noted to the valine α-amino group of $N^{\epsilon}{}_{19}$-acetimidomyoglobin (DiMarchi, 1978). The high degree of coupling to the des-Val[1],$N^{\epsilon}{}_{19}$-acetimidomyoglobin reflects the solvent accessibility of the NH_2 terminus where static accessibility measurements (Lee & Richards, 1971) predict its subsequent burial upon coupling with the activated valine.

In addition to reaction with the α-amino group, coupling to another site in 17% yield was noted (Figure 5G). The addition of various scavengers, for histidine, methionine, tryptophan, and tyrosine, to the reaction mixture revealed phenol to be the most effective in decreasing the amount of byproduct formed. This infers that the side reaction is at a tyrosine residue. This side reaction interfered with increasing the yield of the N^{α}Msc,$N^{\epsilon}{}_{19}$-acetimidomyoglobin through the addition of larger excesses of the activated ester.

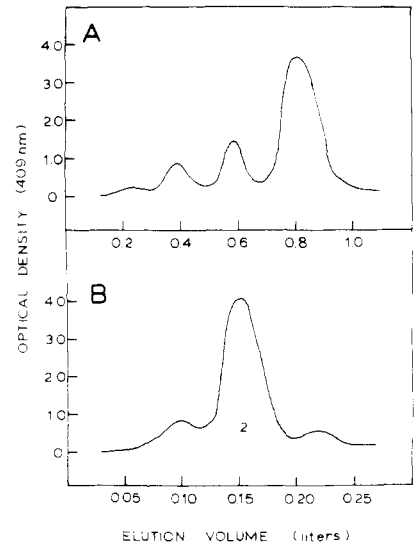

FIGURE 7: The elution profiles of chemically modified myoglobins. Both purifications employed CM-Sephadex C-50 resin with 0.1 μ phosphate, pH 6.45, 25 °C, as the eluting buffer. (A) Products after reconstitution of heme with trifluoroacetic acid treated N^{α}3-sulfo-PhNHCS,$N^{\epsilon}{}_{19}$-acetimidoapomyoglobin and hemochromogen precipitation on a column 4.3 × 53 cm, 80 mL/h. (B) Products after coupling of Msc-valine-ONSu to des-Val[1],$N^{\epsilon}{}_{19}$-acetimidomyoglobin on a column 2.2 × 34 cm, 20 mL/h. The number under each major fraction refers to (1) des-Val[1],$N^{\epsilon}{}_{19}$-acetimidomyoglobin; (2) N^{α}Msc,$N^{\epsilon}{}_{19}$-acetimidomyoglobin.

The N^{α}Msc,$N^{\epsilon}{}_{19}$-acetimidomyoglobin was purified on CM-Sephadex C-50 at pH 6.45 (Figure 7B) and identified by NH_2-terminal sequence analysis (Table I). Electrophoresis revealed the protein to be 93% pure (Figure 5I). Removal of the amino-protecting groups under alkaline treatment was found to be complete by NH_2-terminal sequence quantitation

Table III: Circular Dichroism Analysis of Myoglobin Derivatives

myoglobin	pH	θ_{208}[c] × 10^{-3}	θ_{222} × 10^{-3}	θ_{274} × 10^{-1}	θ_{295} × 10^{-1}	θ_{422} × 10^{-1}	α helix[a]	random coil[a]
myoglobin	6	−17.87	−21.34	23.86	−9.76	78.67	0.71	0.13
	8	−18.04	−21.54	24.18	−9.79	81.76	0.73	0.16
	10.5	−18.27	−21.73	21.77	−9.74	81.35	0.75	0.15
$N^{\epsilon}{}_{19}$-acetimidomyoglobin	6	−18.30	−20.16	24.03	−10.38	88.25	0.67	0.24
	8	−18.46	−20.52	23.82	−9.69	79.94	0.68	0.20
	10	−17.84	−19.46	23.38	−9.85	82.46	0.65	0.26
$N^{\epsilon}{}_{19}$-acetimidoapomyoglobin	6.5	−16.24	−17.07	nd[b]	nd	nd	0.57	0.32
N^{α}3-sulfo-PhNHCS,$N^{\epsilon}{}_{19}$-acetimido-apomyoglobin	6.5	+1.20	−4.98	nd	nd	nd	0.10	0.60
CF_3COOH-treated N^{α}3-sulfo-PhNHCS,$N^{\epsilon}{}_{19}$-acetimido-apomyoglobin	6.5	−6.10	−10.44	nd	nd	nd	0.30	0.47
des-Val^1,$N^{\epsilon}{}_{19}$-acetimidomyoglobin	6	−19.92	−22.69	23.97	−12.53	81.71	0.75	0.15
	8	−19.83	−22.48	23.97	−13.62	83.88	0.75	0.17
	10	−20.34	−23.13	25.06	−16.34	83.88	0.78	0.18
N^{α}Msc,$N^{\epsilon}{}_{19}$-acetimidomyoglobin	6	−17.52	−19.01	20.37	−7.88	68.29	0.64	0.26
	8	−18.50	−20.49	20.35	−9.19	67.62	0.68	0.22
	10	−18.67	−20.62	20.79	−9.10	70.17	0.70	0.26
semisynth myoglobin	6	−19.75	−22.23	25.74	−9.53	77.22	0.75	0.22
	8	−19.55	−21.89	24.62	−9.47	74.81	0.74	0.24
	10	−19.20	−21.39	23.73	−9.89	68.57	0.72	0.24

[a] The amounts of α helix and random coil were calculated by the method of Chen et al. (1972) and are reported as a percent fraction of unity. [b] nd represents not determined. [c] θ is given in units of deg cm^2 dmol^{-1}.

and amino acid analysis (Table I). The semisynthetic protein was shown to move isoelectrically with virgin myoglobin by electrophoresis (Figure 5A,J).

Analysis of the Synthetic Procedure. The overall yield for selective removal and replacement of the NH_2-terminal valine was 0.31% (Table I). The yield-limiting step was found to be the removal of the NH_2-terminal residue from the $N^{\epsilon}{}_{19}$-acetimidomyoglobin. Since the conditions of trifluoroacetic acid treatment (Figure 4) and hemin incorporation (Table II) had been eliminated as sources of any deleterious effects, the 3-sulfo-PhNCS coupling step was investigated more closely. Exposure of methylthiohydantoin amino acids to 3-sulfo-PhNCS showed minimal side chain reactivity (DiMarchi, 1978), thereby suggesting a secondary effect by the reagent.

Isolation of the N^{α},$N^{\epsilon}{}_{19}$-acetimidomyoglobin derivative (Wang, 1977; DiMarchi et al., 1978b) provided a molecule in which all amino groups were incapable of reacting with the 3-sulfo-PhNCS reagent. Electrophoresis[2] of the cyanoferri-N^{α},$N^{\epsilon}{}_{19}$-acetimidomyoglobin after subjection to the 3-sulfo-PhNCS treatment showed no covalent coupling. However, displacement of the high affinity cyanide ligand was apparent and was confirmed by ultraviolet–visible absorption measurements.[2] It was noted by circular dichroism measurements that increasing additions of 3-sulfo-PhNCS beyond tenfold excess yielded a progressive and irreversible denaturation of the secondary structure in the apoprotein (Figure 8). This finding clarifies the sharp decrease in the θ_{280} and θ_{222} noted for the N^{α}3-sulfo-PhNHCS,$N^{\epsilon}{}_{19}$-acetimidoapomyoglobin (Table III). The normal ellipticity returned only after purification of the aquoferri-des-Val^1,$N^{\epsilon}{}_{19}$-acetimidomyoglobin from irreversibly denatured protein.

Discussion

The successful employment of phenyl isothiocyanate in sequential determinations of the primary structure of polypeptide (Edman & Henschen, 1975) has led to its use in protein modification studies (Africa & Carpenter, 1970; Borras & Offord, 1970). Through elegantly designed experiments centering on the use of phenyl isothiocyanate, a detailed analysis of the structural significance of the NH_2-terminal residue of the A and B chains of insulin was completed (Brandenberg et al., 1975; Saunders & Offord, 1977; Geiger et al., 1978). Additional semisynthetic studies (Lode et al., 1974; Slotboom & deHaas, 1975; Kowalski & Laskowski, 1976; Harris & Offord, 1977) have shown that reaction in the presence of organic solvents with all ϵ-amino groups previously protected facilitates the formation of the N^{α}-PhNHCS derivative. The organic solvents improve the solubility and minimize the hydrolysis of the reagent. By virtue of its greater water solubility, 3-sulfo-PhNCS was found to increase the coupling yield in aqueous solvent mixtures (Garner, 1974; DiMarchi et al., 1978a). The subsequent cleavage of the PhNHCS amino acid requires an acid-stable ϵ-amino-protecting group. Selective reversible protection of the ϵ-amino groups of myoglobin through reaction with methyl acetimidate can be achieved in high yield with little structural perturbation (Garner, 1974; Ohms, 1976; Gurd et al., 1977; DiMarchi et al., 1978b).

In this study 3-sulfo-PhNCS coupled to 96% of the NH_2 terminus selectively and the derivative cleaved in trifluoroacetic acid yielding 73% as the des-Val^1,$N^{\epsilon}{}_{19}$-acetimidoapomyoglobin (Table I). Unfortunately less than 10% of this product was isolated with the correct stereochemistry because of the denaturing effects of the reagent upon the acetimidomyoglobin structure. Comparison of the physical properties of the final semisynthetic myoglobin with freshly purified untreated myoglobin through circular dichroism (Table III), potentiometric titration (Figure 9), and ultraviolet–visible absorption measurements (Table IV) showed that the two molecules were identical. The slight differences noted can be accounted for by the small impurity seen in electrophoresis (Figure 5J). Consequently any structural changes observed in the synthetic intermediates can be judged as characteristic of the molecule.

The primary structure of all synthetic intermediates was ascertained through amino acid analysis, NH_2-terminal sequence quantitation, and electrophoresis. Once the identity

Table IV: Ultraviolet-Visible Extinction Coefficient Ratios for Acetimidomyoglobin Derivatives

ligand	wavelength (nm) absorbance ratio	myoglobin[a]	$N^{\epsilon}{}_{19}$-acetimido-myoglobin	des-Val^1,$N^{\epsilon}{}_{19}$-acetimido-myoglobin	N^{α}Msc,$N^{\epsilon}{}_{19}$-acetimido-myoglobin	semisynth myoglobin
H_2O	409/280	5.33	5.18	4.95	4.97	5.15
CN	423/280	3.48	3.56	3.39	3.41	3.49
	360/280	0.91	0.92	0.88	0.91	0.90
	541/280	0.34	0.34	0.33	0.34	0.33
	280(CN)/280(H_2O	1.02	1.02	1.00	1.01	1.02
CO	542/579	1.15				1.10
	345/579	2.20				2.40

[a] Wavelength absorbance ratios as determined by Nakhleh (1971) and Hardman et al. (1966).

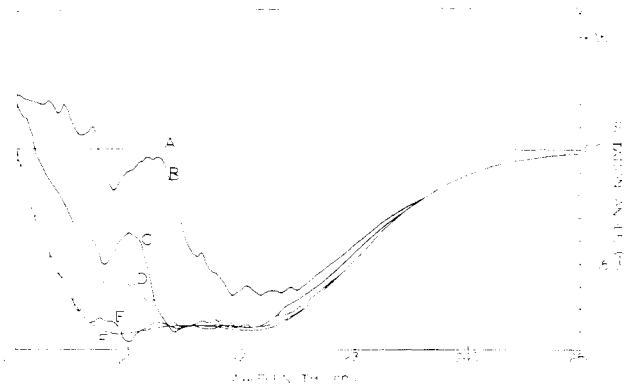

FIGURE 8: Far-ultraviolet circular dichroism spectrum of N^{α},$N^{\epsilon}{}_{19}$-acetimidoapomyoglobin at pH 6.0 illustrating the effects of excess addition of 3-sulfo-PhNCS. (A) 100-fold equivalent of 3-sulfo-PhNCS in water at pH 6.0 without protein; (B) 25-fold excess to protein; (C) 20-fold; (D) 15-fold; (E) 10-fold; (F) 0- to 5-fold.

and homogeneity of each molecule was established, its molar ellipticity and ultraviolet–visible absorption spectra were measured. From these results, several interesting features of the myoglobin molecule became apparent. The absorbance ratio ϵ_{409}:ϵ_{280} is known to be a sensitive monitor of the intact structure of the protein (Breslow & Gurd, 1962; Adler et al., 1973). Table IV supports this finding again since modification as far removed from the heme as the NH_2 terminus (Takano, 1977) still elicits a noticeable change.

It is interesting that substitution of acetimidolysine for all 19 lysines results in only a small change in the ϵ_{409}:ϵ_{280} absorbance ratio. The near- and far-ultraviolet circular dichroism spectra of the $N^{\epsilon}{}_{19}$-acetimidomyoglobin and the unmodified myoglobin are nearly indistinguishable (Table III). The similarity in their spectral properties is probably due in part to the nearly even distribution of lysines over the myoglobin surface as well as to the retention of positive charge. This close identity suggests the elimination of the deacetimidation step as obligatory in subsequent myoglobin semisynthetic studies (Hagenmaier et al., 1978).

In contrast to the far-ultraviolet circular dichroism, which provides an overall view of the molecular structure, the near-ultraviolet provides a means of analyzing specific regions. Nicola et al. (1975) have assigned the 295-nm band in myoglobin to the ^{1}La O–O hydrogen-bonded tryptophan transition and pointed out the absence of any direct heme–tryptophan CD coupling contribution at this wavelength. Since the two tryptophans reside in the A helix (residues 7 and 14), the effects of modification in the NH_2-terminal region would most likely be reflected in the 295-nm band. Perturbations occurring exclusive of the A helix can be recognized by observation of the 274- and 422-nm bands which reflect tyrosine–heme interactions (Strickland et al., 1970) and the asymmetric environment of the heme (Nicola et al., 1975),

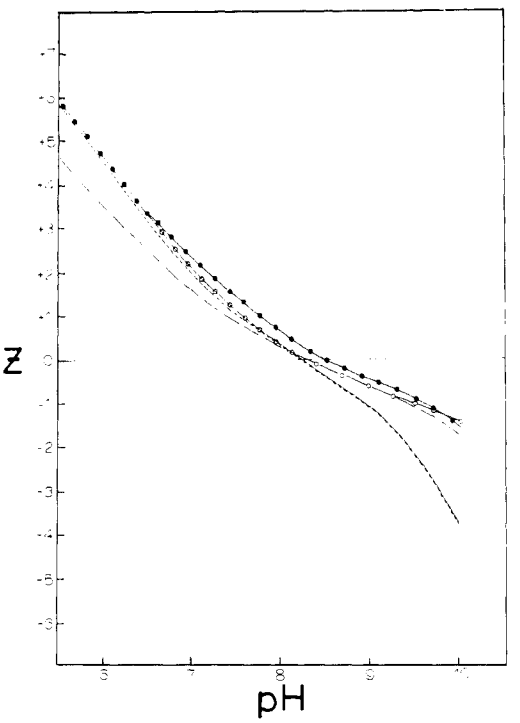

FIGURE 9: Potentiometric hydrogen ion titration curves of the acetimidomyoglobin semisynthetic derivatives. The titration curve of myoglobin is presented for comparative purposes. The horizontal line in the middle is the reference position of zero net charge. Myoglobin (—); $N^{\epsilon}{}_{19}$-acetimidomyoglobin (O—O); N^{α}Msc,$N^{\epsilon}{}_{19}$-acetimidomyoglobin (– – –); des-Val^1,$N^{\epsilon}{}_{19}$-acetimidomyoglobin (●—●); semisynthetic myoglobin (- - -).

respectively. Inspection of Table III reveals a significant change in the 295-nm band following removal of the NH_2-terminal valine. Alteration of pH from 6 to 10 yielded uncharacteristic increases in the rotational strength. This observation could result from movement of either tryptophan side chain into juxtaposition with a proximal aromatic site (Strickland, 1974), such as phenylalanine-123 or -138. However, the pH range where the transition was noted suggests the involvement of the NH_2-terminal leucine residue. The formation of a hydrogen bond between the unprotonated leucine α-amino group and the indole nucleus of tryptophan-7 would account for the increased tryptophanyl CD intensity (Strickland, 1974) and be in accord with the atomic coordinates of the molecule (Takano, 1977).

The effects of modifications at the NH_2 terminus upon the net charge of the protein at various pH values were assessed through potentiometric titration (Figure 9). Removal of the NH_2-terminal valine of $N^{\epsilon}{}_{19}$-acetimidomyoglobin led to an increased net charge observed between pH 6.5 and 9.5. Since the total content of charged amino acids was not changed through this cleavage, it seems reasonable to suspect an in-

creased pK for the new α-amino group. This could arise in part from the NH_2 terminus now being located farther away from the positively charged lysine-133 and histidine-81 (Takano, 1977). Attachment of the Msc-L-valine to the des-Val[1] protein yielded a myoglobin with a nontitrating NH_2 terminus. Consequently, the N^{α}Msc,N^{ϵ}_{19}-acetimidomyoglobin and N^{ϵ}_{19}-acetimidomyoglobin appear nearly identical until the α-amino group begins to protonate. At pH 7.2 ± 0.1, they differ by one-half charge and this should identify the apparent pK of the NH_2-terminal valine. The presence of the acetimidolysines probably has also contributed to a decreased pK for the α-amino group as compared with the 7.96 of unmodified sperm whale myoglobin (Garner et al., 1973).

In future semisynthetic experiments, the use of 3-sulfo-PhNCS will be for those polypeptides which require coupling in aqueous media and are immune to its particular denaturating effects. An attractive alternative to 3-sulfo-PhNCS for myoglobin is methyl isothiocyanate (Stepanov & Krivtsov, 1965). In the present work, optimum conditions for removal of the NH_2-terminal residue of acetimidoapomyoglobin in trifluoroacetic acid were developed along with an improved method of reheming its structurally altered conformers. Acetimidyl protection of the ϵ-amino groups proved to be extremely stable to the trifluoroacetic acid cleavage conditions. This was critically important since even 1% deacetimidation would have yielded approximately 20% of the resultant des-Val[1]-acetimidomyoglobin with less than complete protection.

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Supplementary Material Available

Additional data on amino acid analysis, aggregation, trifluoroacetic acid treatment, electrophoresis, and ultraviolet–visible absorption measurements (8 pages). Ordering information is given on any current masthead page.

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