Characterization of the Reaction of Methyl Acetimidate with Sperm Whale Myoglobin†

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ABSTRACT: The effects of pH, acetimidate concentration, temperature, and reaction time of methyl acetimidate with sperm whale myoglobin have been assessed. Reaction at pH 9.8 and 15 °C for 30 min with a sixfold excess of methyl acetimidate relative to each amino group yielded six acetimidomyoglobin derivatives which were separated and purified. Reaction with tetrahydrophthalic anhydride revealed the number of amino groups that remained unreacted in each separated component and made possible further subfractionation. Modification at the NH_2 terminus was quantitated by automated stepwise Edman degradation. The acetimidyl and tetrahydrophthalyl groups were readily removable. The potentiometric titration of three of the completely deprotected components showed identity with the parent untreated sperm whale myoglobin. The first of two major products was acetimidated at all 19 ϵ-amino groups but not at the NH_2 terminus. The second major product bore a blocked NH_2 terminus but retained one unmodified ϵ-amino group, identified after modification by trinitrobenzenesulfonate as lysine residue 77. Of the minor components, one was identified as completely acetimidated at all 20 amino groups. The other three minor components appeared to contain irreversible by-products.

The specific reconstruction of proteins from natural biological intermediates (semisynthesis) provides a method for investigating the structure–function relationships of macromolecules. In this way the sequence may be altered or structurally nonperturbing probes may be incorporated into the primary structure with a minimum possibility of error. Potentially, appropriate semisynthetic products could be used to probe almost any question concerning the function, stability, or dynamics of a protein.

The main obstacles to use of naturally occurring polypeptide intermediates have been the requirements for reversible protection of functional groups and of cleavage methods and recoupling techniques that do not impose conditions of irreversible denaturation upon the protein. For most proteins media of high polarity are required during all semisynthetic manipulations, while strictly nonaqueous conditions will be permissible only for certain steps.

Semisynthetic strategy has been divided into the two approaches of stepwise and fragment condensation (Offord, 1973) depending on whether the initial cleavage of the natural peptide entails the sequential removal of one or more amino acids from an end (NH_2 terminus or COOH terminus) or the splitting of the chain at some internal point down its length. In either case one or more fragments derived from the initial protein will need to be selectively protected before the recoupling of an amino acid or peptide component can be achieved.

This report describes the use of the acetimidyl group for reversible protection of amino groups in sperm whale myoglobin. Acetimidyl protection has the advantage (Hunter & Ludwig, 1962) that it retains the native charge of the ϵ-amino groups while changing pK values only slightly. The presence of the native charge pattern maintains water solubility while the limited change in the isoionic point preserves the stability of the protein. This is extremely important when working with a protein such as myoglobin which is susceptible to irreversible denaturation in nonaqueous media (Herskovits & Solli, 1975; Herskovits et al., 1977). The acetimidyl protective group has the further advantage that reaction can be directed preferentially to the ϵ-amino groups (Hunter & Ludwig, 1962), producing some of the product in the form that retains a free α-amino group while bearing the acetimidyl group on all lysine side chains (Garner & Gurd, 1975).

The application to myoglobin has general value since this protein contains 19 lysine residues in various environments on its surface and provides a good test of the suitability of methyl acetimidate as a reversible protecting agent compatible with subsequent cleavage and coupling strategies. The importance of this application is perhaps most clearly foreshadowed by the valuable results obtained with ferredoxins (Hong & Rabinowitz, 1970; Lode et al., 1974, 1976) and insulin (Borras & Offord, 1970; Africa & Carpenter, 1970; Geiger et al., 1975; Krail et al., 1975) in which at most one ϵ-amino group required protection. In these cases both stepwise and fragment condensations could be employed with success since the strongly nucleophilic ϵ-amino group, if present, could be protected in a relatively selective manner and the requirement for a derivative conferring water solubility was not great.

The present report describes the products of treatment of myoglobin with methyl acetimidate and the separation of derivatives containing acetimidyl groups protecting only the 19 ϵ-amino groups in one case and both the 1 α- and the 19 ϵ-amino groups in another. These products are suitable, respectively, for stepwise and fragment cleavage and reconstruction procedures. In addition a product containing acetimidyl protection at the α-amino group and at 18 of the ϵ-amino groups has been characterized to show the preponderance of a specific lysine residue that remains unmodified. Finally, the

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production and removal of small quantities of protein bearing nonamidine reaction products are reported.

Experimental Section

Materials. 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 all studies. The minor natural components III and II were studied for reference. Methyl acetimidate was prepared according to the procedure of Hunter & Ludwig (1962). All reagents and buffers (pico buffer system II) for the amino acid analyzer were purchased from Pierce. The reagents for amino acid sequence analysis were sequencer grade reagents from Beckman. Poly(L-lysine), poly(D,L-alanine) was purchased from Miles. Carboxypeptidase Y was acquired from Pierce, aminopeptidase M from Sigma, and staphylococcal protease from Miles. The urea was recrystallized from ethanol and deionized before use. All other chemicals were reagent grade quality.

NH_2-Terminal Analysis. The NH_2-terminal amino acid was identified quantitatively by subjecting the apoprotein derivatives to several degradative cycles on the Beckman 890C Sequencer. Typically, 10 mg (600 nmol) of protein was dissolved in 300 μL of glacial acetic acid and 300 μL of a 20 mM stock solution of a multichain poly(amino acid) polymer. The multichain macromolecule (mol wt $\simeq$ 80 000) consisted of a poly(L-lysine) core which had D,L-alanine polymerized to the ϵ-amino groups in a ratio of 13.2:1 poly(D,L-alanine):L-lysine. The purpose of introducing the heteroamino acid polymer to the sequencer spinning cup was to provide an internal standard of comparison within a given series of degradative cycles. A 30-μL aliquot of the solution which was applied to the spinning cup was also subjected to amino acid analysis to determine the actual protein concentration. The 1-chlorobutane was removed by a stream of nitrogen on a N-EVAP evaporator (Organomatic Association Inc.) and the sample hydrolyzed in a solution containing 2 mL of 5.7 N HCl and 5 μL of mercaptoethanol at 150 °C (Van Orden & Carpenter, 1964). These samples were flash evaporated and applied to the amino acid analyzer. The mole fraction of free NH_2 terminus was calculated by dividing the recovered amino acid terminal residue by the total amount of protein present in the reaction cup. The remaining mole fraction was attributed to an acetimidyl blocked NH_2 terminus.

Sequencing Techniques. The Beckman fast protein Quadrol program (072172C) was used to sequence the NH_2-terminal region of the larger peptides, while those consisting of 25 residues or less were sequenced with the fast peptide-DMAA[1] program (102974).

Amino Acid Analysis. The protein hydrolysates were prepared according to the procedure of Spackman et al. (1958) and analyzed as reported previously (Jones et al., 1978). Amino acid composition of the ferrimyoglobin acetimidated derivatives was determined from 24-, 48-, and 72-h hydrolysates (Dwulet et al., 1975; Garner & Gurd, 1975). The quantity of acetimidolysine present was determined by extrapolation to zero time.

Electrophoresis. The homogeneity of all the myoglobin preparations was established by electrophoresis on cellulose acetate strips at 300 V, in 0.1 M Tris-EDTA-borate (pH 9.2) and/or in 0.05 μ phosphate buffer (pH 6.5), followed by staining with Ponceau S (Beckman fixative dye) and scanning at 530 nm with a Beckman Analytrol densitometer.

Ultraviolet and Visible Absorption Measurements. Spectra measured with a Cary Model 14 spectrophotometer were converted to protein concentrations using $\epsilon_{409} = 16.8 \times 10^4$ and $\epsilon_{280} = 3.15 \times 10^4$ M^{-1} cm^{-1} (Nakhleh, 1971), and the absorbance ratio ϵ_{409}:ϵ_{280} was employed as a sensitive monitor of the intact structure of the myoglobin molecule (Breslow & Gurd, 1962; Adler et al., 1973).

Isoionic Points and Titration Curves. Isoionic points and potentiometric hydrogen ion titration curves of sperm whale ferrimyoglobin and its acetimidated derivatives were determined as previously described (Nakhleh, 1971; Shire et al., 1974). The isoionic point was taken as the pH of deionized protein solution at 25.0 °C and at a concentration of about 200 μM. It was measured in a thermostated cell compartment of a Radiometer PH M4 null reading potentiometer, the protein being passed under nitrogen from a deionizing column directly into the cell.

The same apparatus was used in the potentiometric determinations of titration curves, except that an aliquot of KCl was added to maintain a constant ionic strength of 0.01 M. Two titrations were carried out in every case: one starting from the pH of isoionic protein in KCl and moving down by addition of 0.1 N HCl, and the other again starting from the pH of isoionic protein in KCl but moving up with 0.1 N NaOH. Separate duplicate blank titrations of equal volumes of 0.01 M KCl solution were also conducted with the acid and with the base. Protein concentrations were determined by converting myoglobin samples into the cyanoferrimyoglobin derivative. The absorption spectrum of this low-spin stable complex has been shown to be identical among the different natural components of sperm whale myoglobin (Nakhleh, 1971), and it was therefore used as a basis for determining concentrations of the acetimidated derivatives, with $\epsilon_{541} = 1.08 \times 10^4$ M^{-1} cm^{-1}. This enables the determination of the number of moles of hydrogen ion bound per mole of myoglobin as a function of pH; since the isoionic point for ferrimyoglobin is not far from pH 7, it serves as the reference point of zero net protein charge.

Reaction of Methyl Acetimidate with Sperm Whale Myoglobin. Acetimidation of sperm whale myoglobin was accomplished under various conditions by the following general procedure. A 3% solution of purified sperm whale ferrimyoglobin was cooled to the desired temperature and its pH adjusted to the reaction pH with 0.1 N NaOH. The methyl acetimidate hydrochloride was adjusted to the reaction pH with cold 5 N NaOH prior to addition to the protein. Throughout the reaction the pH was maintained by the addition of 1.0 M NaH_2PO_4. The reaction was terminated by gel filtration on a Sephadex G-25 (medium) column equilibrated with 0.1 M tris(hydroxymethyl)aminomethane (pH 9.4) at 4 °C.

Reaction of 3,4,5,6-Tetrahydrophthalic Anhydride with Acetimidomyoglobin. A 3% solution of acetimidated ferrimyoglobin was allowed to react with a tenfold excess of 3,4,5,6-tetrahydrophthalic anhydride at pH 7.4, 16 °C for 3 h (Gibbons & Schachman, 1976). The pH was maintained by the addition of 2 N NaOH. Upon completion of reaction, the protein was dialyzed for 24 h against water at 4 °C. The tetrahydrophthalyl group served as a charged, hydrophilic handle for the chromatographic isolation of the desired acetimidomyoglobin derivatives. The tetrahydrophthalyl groups were subsequently removed by dialysis against 0.1 M sodium phosphate (pH 6.0) for 30 h at room temperature.

Removal of the Acetimidyl Group. The acetimidyl groups were removed by exposure of the holoprotein derivative to

[1] Abbreviations used: THP, tetrahydrophthalyl; TNBS, trinitrobenzenesulfonate; Pth, phenylthiohydantoin; Tos-PheCh$_2$Cl, L-1-tosylamido-2-phenylethyl chloromethyl ketone; DMAA, dimethylallylamine; AT-Lys, acetimidolysine.

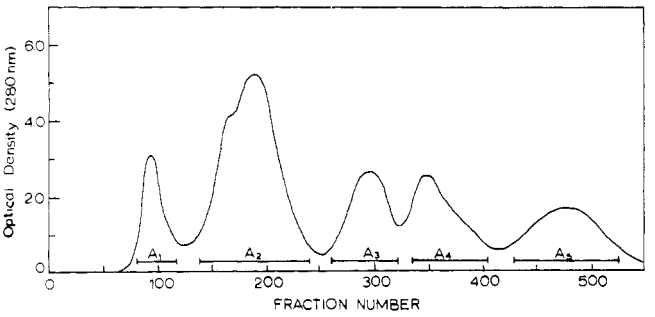

FIGURE 1: Separation pattern of the acetimidomyoglobin derivatives on a DEAE-A50 Sephadex column (9.5 × 50 cm) in 0.05 M Tris buffer (pH 8.9) at 4 °C after reaction at pH 9.8, 30 min, 15 °C, with a 120-fold excess of methyl acetimidate. The flow rate was maintained at 400 mL/h and 15 mL of eluent was collected in each fraction tube. The fractions were combined in pools as indicated.

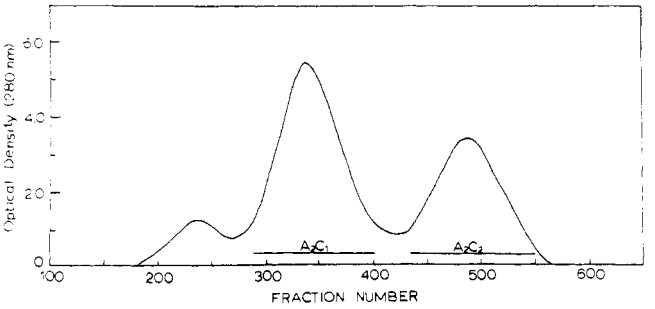

FIGURE 2: Ion-exchange elution pattern of the acetimidomyoglobin derivative A_2 on a CM-C50 Sephadex column (7.5 × 50 cm) in 0.1 μ phosphate buffer at pH 6.5. The flow rate was maintained at 250 mL/h and 15 mL of eluent was collected in each fraction tube.

concentrated ammonium hydroxide-acetic acid (15:1, v/v) at pH 11.5, 16 °C (Ludwig & Byrne, 1962; Reynolds, 1968). Under these conditions approximately 4 h was necessary for removal of 50% of the acetimidyl groups, and so a period of at least 28 h was allowed. After ammonolysis, the deacetimidated protein was exhaustively dialyzed at 4 °C against 10 mM sodium phosphate buffer (pH 6.5).

Reaction of Trinitrobenzene Sulfonate with Acetimidomyoglobin. A 20-mg sample (1.1 μmol) of acetimidated apomyoglobin was dissolved in 10 mL of 4 M urea. The solution was adjusted to be 0.05 M in borate by the addition of 190 mg of sodium tetraborate decahydrate which brought the pH to 9.6, at 25 °C. One milliliter of an aqueous trinitrobenzenesulfonate solution (7.2 mg/mL) was added to the protein and the reaction was allowed to proceed for 12 h (Habeeb, 1966) in the dark. The protein was dialyzed extensively against water and then 10% acetic acid for an additional 6 h.

Cyanogen Bromide Cleavage of Acetimidomyoglobin. The protein was dehemed as described by Yonetani (1967) and cleaved with cyanogen bromide according to the method of Dwulet et al. (1975).

Tryptic Cleavage of the Acetimidated Peptide 56–131. To 30 mg (3.4 μmol) of the cyanogen bromide cleavage peptide 56–131 dissolved in 5 mL of 5 M urea was added 0.6 mg (2% w/w) of trypsin (Tos-PheCH_2Cl-treated, Worthington). The pH was adjusted to 8.0 with 0.1 N NaOH and the temperature maintained at 25 °C. After 3 h another 0.6 mg of trypsin was added and the reaction was stopped by lowering the pH to 3.0 with acetic acid. The digest was desalted on a Bio-Gel P-2 column.

Staphylococcal Protease Cleavage of the Acetimidated Peptide 56–131. The acetimidated peptide 56–131 was cleaved specifically at glutamic acid residues with staphylococcal protease according to the procedure of Bogardt et al. (1976).

Enzymatic Digestion of the Acetimidated Peptide 132–153. To 4 mg (1.6 μmol) of the cyanogen bromide cleavage peptide 132–153 dissolved in 1 mL of 0.05 M sodium citrate buffer (pH 5.7) was added 0.35 mg of carboxypeptidase Y (Hayashi et al., 1973). After 8 h at 37 °C the pH was adjusted to 8.0 with 30 μL of 1 N NaOH. Aminopeptidase M, 0.5 unit (Wachsmuth et al., 1966), was added and digestion was allowed to proceed for an additional 10 h at 37 °C. The reaction was terminated with the addition of 0.4 mL of Stand In buffer (pH 2.2).

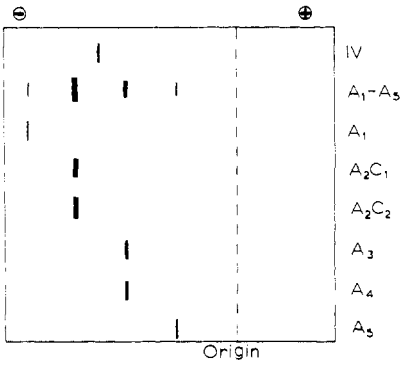

FIGURE 3: Electrophoretic separation of the acetimidomyoglobin derivatives on cellulose acetate in 0.1 M Tris-EDTA-borate (pH 9.2). The acetimidyl reaction product before purification is denoted as A_1-A_5.

Results

Isolation of Acetimidyl Derivatives. The products of the acetimidation reaction were separated on DEAE-A50 Sephadex in 0.05 M Tris buffer (pH 8.9) at 4 °C. Figure 1 shows the separation achieved and the system for naming fractions A_1 to A_5. Each fraction was dialyzed exhaustively, first against water and then pH 6.5, 0.1 μ phosphate buffer, and purified on CM-C50 Sephadex. The pH of these columns was 6.9, 6.5, 6.3, 6.3, and 6.1 for A_1, A_2, A_3, A_4, and A_5, respectively. With the exception of fraction A_2, all the columns yielded one major component. Fraction A_2 divided into two distinct bands at pH 6.5 that are designated in Figure 2 as A_2C_1 and A_2C_2, respectively. The six components were dialyzed against water at 4 °C. Electrophoresis at pH 6.5 and pH 9.2 showed each component to be homogeneous as illustrated for pH 9.2 by Figure 3. The separation of these components revealed an ordered pattern, with A_1 proving one step more positive than A_2C_1 or A_2C_2, two more than A_3 and A_4, and three more than A_5. The following results show that components A_1, A_2C_1, and A_2C_2 hold the main interest for semisynthetic manipulations.

The potentiometric titration of these derivatives (Figure 4) reveals that at high pH all the derivatives are more positive than sperm whale myoglobin fraction IV, the major myoglobin component and starting material for this work. This distinction may be attributed to a rise in pK of the lysine residues as a result of acetimidation. However, the acid range of the titration curves (Figure 4) is more informative since components A_1, A_2C_1, and A_2C_2 align with the natural sperm whale myoglobin component IV, A_3 and A_4 coincide with the natural minor component III (not shown), and A_5 aligns with the natural minor component II. The minor components III and II are 1 and 2 charges, respectively, more negative than the major IV (Hardman et al., 1966; Nakhleh, 1971). These results suggest that the last three acetimidyl components, unlike the first three,

TABLE I: Amino Acid Composition[a] and N-Terminal Analysis[b] of the Acetimidated Derivatives.

amino acid	Mb	A_1	A_2C_1	A_2C_2	A_3	A_4	A_5
Asp	8	7.9	7.9	7.8	7.8	7.5	7.7
Thr	5	4.8	4.5	4.7	4.8	4.9	4.5
Ser	6	5.5	5.2	5.3	6.0	5.7	5.6
Glu	19	20.5	20.7	21.3	20.1	19.2	19.6
Pro	4	4.0	4.2	4.2	4.1	4.0	4.2
Gly	11	10.9	11.0	10.8	11.1	10.5	10.9
Ala	17	17.7	17.4	18.0	18.5	18.5	18.6
Val	8	7.9	8.0	7.9	7.5	7.8	8.3
Met	2	2.3	1.6	2.3	1.9	2.1	2.4
Ile	9	8.3	8.5	8.6	8.8	9.1	8.8
Leu	18	18.8	18.4	18.3	18.5	18.1	18.0
Tyr	3	2.6	2.7	2.4	2.6	3.0	3.3
Phe	6	6.4	5.5	5.5	5.4	6.1	6.6
AT-Lys	19	18.9	18.4	18.7	17.4	16.9	14.9
His	12	12.0	12.8	11.5	12.7	13.0	12.9
Arg	4	2.9	3.8	3.2	3.5	3.9	4.1
Trp[c]	2	1.8	1.9	2.1	2.0	1.7	2.2
sequencer cycle	Mb	A_1	A_2C_1	A_2C_2	A_3	A_4	A_5
1	Val	Val	Val	Val	Val	Val	Val
mole fraction	1.00	0.00	0.03	0.96	0.86	0.72	0.83
2	Leu	Leu	Leu	Leu	Leu	Leu	Leu
mole fraction	1.00	0.06	0.00	0.89	1.02	0.70	0.78

[a] Protein hydrolysis was performed for 24, 48, and 72 h. Serine, threonine, and acetimidolysine content were determined by extrapolation to zero time while isoleucine and valine contents were determined by extrapolation to 100 h. [b] The yield reported under sequencer cycle is that fraction of N-terminal remaining unreacted. [c] Tryptophan was determined by the method of Liu & Chang (1971).

have incurred some type of additional or alternative modification.

Time course amino acid analyses were conducted to determine the extent of lysine side chain protection (Table I). Components A_1 and A_2C_2 showed substantially complete conversion to acetimidolysine, while A_2C_1, A_3, and A_4 showed somewhat less than complete acetimidation. Component A_5 reveals limited reaction, with at least 4 equiv of lysine failing to appear in the acetimidolysine form. Included in Table I are the NH_2-terminal sequence analyses. Within experimental error A_1 and A_2C_1 are completely blocked at the NH_2 terminus, while A_2C_2 is completely free. Components A_3, A_4, and A_5 show some reaction at the NH_2 terminus, an indication that these components are not completely homogeneous.

Components with Complete Lysine Acetimidation. The two components of immediate value for semisynthesis (Gurd et al., 1977) were the completely blocked form, A_1, and the form with only the α-amino group unprotected, A_2C_2 (Table I). These two components were subjected to ammonolysis to remove the acetimidyl groups. In both cases the product was indistinguishable from the native fraction IV by gel electrophoresis, amino acid composition (see paragraph concerning supplementary material at the end of this paper) and titration (Figure 5).

Component with Complete Acetimidation Except for One Lysine Residue. Fraction A_2C_1 (Figures 2, 4, and 5, Table I) differed from the form with only the α-amino group unprotected in its chromatographic and titration behavior and, less conclusively, in content of acetimidolysine. Ammonolysis to remove acetimidyl groups yielded a protein indistinguishable from the native fraction IV (Figure 5). The Edman degradation analysis (Table I) showed that the acetimidated preparation had a blocked NH_2 terminus. To test for the presence of unmodified lysine, this material and all other acetimidation reaction products were subjected to tetrahydrophthalic anhydride. Fraction A_2C_1 was converted into two major components. The more positive component on electrophoresis (Figure 6) moved with one additional negative charge attributable to the tetrahydrophthalic anhydride treatment, and the other with three additional negative charges.[2] These two components were separated on CM-C50 Sephadex at pH 6.4,

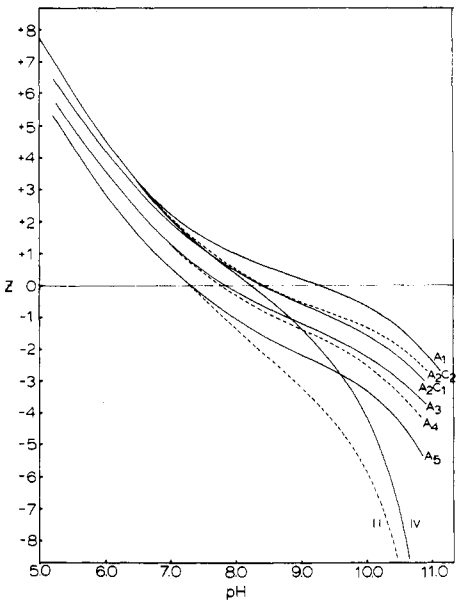

FIGURE 4: Potentiometric hydrogen ion titration curves of the acetimidomyoglobin derivatives. The titration curves of the major (IV) and a minor (II) component of untreated sperm whale myoglobin are presented for comparative purposes. The horizontal line in the middle is the reference position of zero net charge.

[2] At pH 9.2 a normal ϵ-amino group is predominately protonated and the addition of the negatively charged tetrahydrophthalyl group should yield a net charge change of -2.

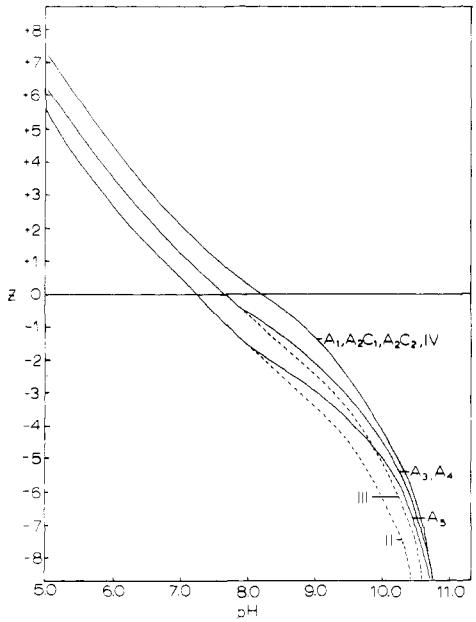

FIGURE 5: Potentiometric hydrogen ion titration curves of the acetimidomyoglobin derivatives after removal of the acetimidyl group. The titration curves of the major (IV) and minor (II and III) components of sperm whale myoglobin are given for comparative purposes. No separation was observed for the titration curves of untreated sperm whale myoglobin IV and the deacetimidated derivatives A_1, A_2C_1, and A_2C_2.

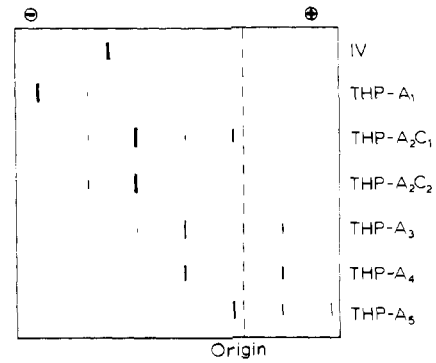

FIGURE 6: Electrophoretic separation (electrophoretic conditions are given in Figure 3) of the tetrahydrophthalic anhydride treated acetimidomyoglobin derivatives.

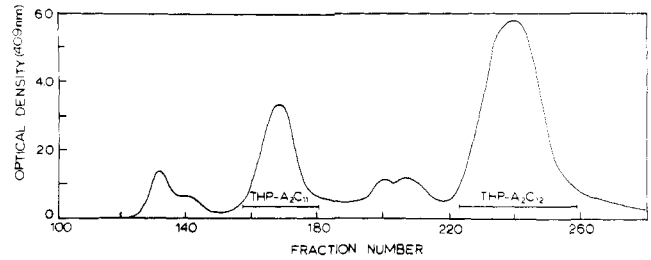

FIGURE 7: Ion-exchange elution pattern of the tetrahydrophthalyl derivatives of A_2C_1 on a CM-C50 Sephadex column (7.5 × 50 cm) in 0.1 μ phosphate buffer at pH 6.4. The flow rate was maintained at 250 mL/h and 15 mL of eluent was collected in each fraction tube.

0.1 μ phosphate buffer at 4 °C (Figure 7). The tetrahydrophthalyl group was removed from the more positive and plentiful component, A_2C_{12}, and the protein dialyzed against water at 4 °C. The product was electrophoretically homogeneous.

Two approaches were made to the identification of the free lysine residue in the A_2C_{12} component. In the first the myoglobin derivative was dehemed (Yonetani, 1967) and treated with trinitrobenzenesulfonate in 4 M urea (Habeeb, 1966). The yellow protein derivative was cleaved with cyanogen bromide and the products isolated on G-50 Sephadex in 10% acetic acid (supplementary material). The yellow color was retained in the cyanogen bromide peptide 56–131 (DiMarchi et al., 1978). This peptide was lyophilized and cleaved with staphylococcal protease (Bogardt et al., 1976). After separation on a phosphocellulose column and amino acid analysis of the pools, peptide 60–83 was identified as the carrier of the trinitrophenyllysine by its yellow color and the absence of one acetimidolysine residue. Upon sequence analysis lysine-77 was found in 34% of the normal sequential yield. Evidently some of the trinitrophenyllysine was converted to free lysine by the hydrolysis preparatory to amino acid analysis.

Confirmation of the free lysine at position 77 was obtained directly from cyanogen bromide cleavage of the A_2C_{12} component without prior treatment with trinitrobenzenesulfonate. The cyanogen bromide peptide 56–131 was subjected to tryptic cleavage and the tryptic peptides were isolated on G-50 Sephadex in 10% acetic acid (supplementary material). Peptides 78–118 and 56–77 were recognized by amino acid analysis. The compositions of all peptide products (supplementary material) and the satisfactory yield of the tryptic cleavage product at residue 77 establish the identity of residue 77 as the dominant unmodified lysine in the acetimidation product.

The electrophoretic behavior of the other acetimidation products on treatment with tetrahydrophthalyl anhydride is shown in Figure 6. The fully acetimidated component A_1 was unaffected. The component A_2C_2 with only the α-amino group free underwent the expected decrease of charge by 1 unit at the pH of 9.2. Edman analysis showed that the α-amino was no longer free but could be regained after removal of the tetrahydrophalyl group.

Irreversible Products of Methyl Acetimidate Treatment. The products of the original methyl acetimidate treatment, A_3, A_4, and A_5, were shown in Figure 5 after removal of acetimidyl groups to have characteristics of proteins bearing higher net negative charges than the original component IV or the reversibly modified components discussed above. These observations could result from either the conversion of an amide to an acid side chain or an irreversible modification of one or more lysine residues. There would be intrinsic interest in a deamidated derivative in particular.

There are 6 possible sites of deamidation in sperm whale myoglobin component IV: glutamine 8, 26, 91, 128, and 152, and asparagine 132 (Edmundson, 1965).[3] Component A_3 was cleaved with cyanogen bromide, after which peptides 1–55 and 132–153 were sequenced to reveal the presence of glutamine 8, 26, and 152, and of asparagine 132 (supplementary material). Tryptic cleavage of cyanogen bromide peptide 56–131, followed by G-50 Sephadex chromatography, yielded the peptides 56–118 and 119–131 which on sequencing confirmed the presence of glutamine 91 and 128.[3] To avoid any systematic partial conversion of the amide to the parent acid, enzymatic digestion of the cyanogen bromide peptide 132–153 was accomplished. Carboxypeptidase Y digestion followed by amino peptidase M yielded the two amides, asparagine 132 and glutamine 152, at mole ratios of unity with no increase in the as-

[3] The original sequence of sperm whale myoglobin component IV reported by Edmundson (1965) showed an amide, asparagine, at position 122. Romero-Herrera & Lehmann (1974) suggested aspartic acid at this position. Various cleavage procedures have also yielded aspartic acid at this position (R. A. Bauman, C. C. Wang, B. N. Jones, R. D. DiMarchi, & L. D. Lehman, unpublished results) and in the work reported here only aspartic acid was observed at position 122 in peptide 119–131 isolated from component A_3.

TABLE II: The Effects of Variations[a] in pH, Time, Temperature, and Acetimidate Concentration on the Formation of the Acetimidyl Derivative of the N-Terminus Valine.

	variation of pH			
pH	9.5	10.0	10.5	11.0
yields[b]	0.37	0.29	0.33	0.39
	variation of time			
time (min)	30	60	120	600
yields	0.29	0.43	0.42	0.56
	variation of temp			
temp (°C)	5	15	25	
yields	0.45	0.29	0.27	
	variation of acetimidate concn			
concn acetimidate[c] (molar proportion)	40	120	200	600
yields	0.25	0.29	0.38	0.45

[a] One of the four reaction conditions was varied from the standard conditions of pH 10.0, 30 min, 15 °C, and a 120-fold excess of methyl acetimidate. [b] Yields are given as mole fraction of acetimidyl N-terminal formed. [c] Concentration of acetimidate used is given as the molar excess to the protein.

TABLE III: The Effects of Changes[a] in pH, Time, Temperature, and Acetimidate Concentration on the Relative Yields of Each Acetimidomyoglobin Component.

	A_1	A_2C_1	A_2C_2	$A_3 + A_4$	A_5
pH					
9.5	12[b]	25	13	34	16
10.0	9	19	49	18	5
10.5	10	23	50	17	0
11.0	9	30	50	11	0
time (min)					
30	9	19	49	18	5
60	13	30	26	26	5
120	12	30	25	27	6
600	18	38	12	26	6
temp (°C)					
5	10	35	33	18	4
15	9	19	49	18	5
25	10	17	44	26	3
concn (acetimidate, molar proportion)[c]					
40	9	16	52	23	0
120	9	19	49	18	5
200	13	25	31	25	6
600	15	30	13	31	11

[a] Reaction conditions were as stated in Table IV. [b] Relative yields are given as a percentage of the total quantity of acetimidyl products. [c] Concentration of acetimidate used is given as the molar excess to the protein.

partic or glutamic acid content (supplementary material). The upshot of these experiments was to turn attention from possible deamidation to possible side reactions in the lysine modification process itself.

Effects of Variation in Reaction Conditions. The effects of variation in pH, acetimidate concentration, duration, and temperature of reaction were assessed in terms of the relative reactivity of the α-amino and ϵ-amino groups. The results in Table II describe the mole fraction of NH_2-terminal residue remaining unreacted, as determined by automated Edman degradation, after varying one of the four reaction conditions. Increases in the acetimidate concentration and extended times of reaction yielded the expected increase in the extent of reaction. As the temperature of the reaction was increased, the rate of methyl acetimidate hydrolysis also increased, so that the yield of blocked NH_2 terminus was greater at lower temperatures. The response to changes in pH was more complex, since the two factors which are responsible for the effect operate in direct opposition. As the pH is raised the loss of methyl acetimidate to hydrolysis is lessened (Browne & Kent, 1975) but its relative consumption by reaction with ϵ-amino groups is promoted (Hunter & Ludwig, 1962).

The relative yields of each acetimidyl myoglobin component under the reaction conditions of Table II were estimated by cellulose acetate electrophoresis and are given in Table III. The relative intensities of each band (A_1, A_2, $A_3 + A_4$, A_5) were obtained by optical scanning as explained in the Experimental Section. In each experiment the mole fraction of acetimidated α-amino group closely corresponds (Table I) to the proportion of $A_1 + A_2C_1$, leading to an estimate of the relative proportions of A_2C_1 and A_2C_2 as listed in Table III. It is these three components that are of primary interest for semisynthetic procedures.

The desired derivatives whose production is most easily manipulated can be seen from Table III to be A_1, the fully protected form, and A_2C_2, the form in which the α-amino group alone is unmodified. As the pH is raised the most striking observation is the sharp increase in A_2C_2 formation at the expense of the undesired components ($A_3 + A_4 + A_5$). For certain purposes the derivative A_1 is desired (Wang et al., 1978) and can be prepared at the expense mainly of the yield of A_2C_2 by increased reaction time and acetimidate concentration. Further increases can be obtained through multiple additions of the imidoester.

Discussion

Three of the acetimidyl derivatives are amenable to semisynthetic studies. Derivative A_1, which is protected at the NH_2 terminus and at all 19 lysines, has been used in semisynthesis (Wang et al., 1977, 1978). Derivative A_2C_2 retains only the free α-amino group and so is directly suited to stepwise semisynthetic manipulation at the NH_2 terminus (Garner & Gurd, 1975; Gurd et al., 1977; DiMarchi et al., 1978). The third derivative identified, A_2C_{12}, differs from A_1 in that lysine residue 77 remains unmodified. Although its semisynthetic potential is easily recognized, it has not yet been explored. The observations on the derivative before (Figure 3) and after (Figure 6) treatment with tetrahydrophthalic anhydride indicate that it acquired only one additional negative charge. Therefore the pK of the ϵ-amino group of lysine-77 must be below 9.2 when the other 19 amino groups are acetimidated.[2] This relatively low pK value may correspond with the observed depressed reactivity toward methyl acetimidate.

There are two lines of evidence that indicate that component A_3 is more negative than sperm whale component IV as a result of irreversible modification of one of its lysine residues. First, low values were found for acetimidolysine (Table I) and recovered lysine (supplementary material), and the confirmation of amide placements in isolated peptides eliminated deamidination. Second, titration of the deacetimidated A_3 (Figure 5) shows that its titration curve is more negative than sperm whale component IV at low and intermediate pH values, while at a point above the pK of the ϵ-amino group the two titration curves converge. It is probable that A_4 differs from A_3 only in the site of modification, while A_5 represents a product or products bearing two irreversibly modified lysine residues. While the exact nature of this modification is not clear, it

nonetheless renders these acetimidomyoglobins of little semisynthetic value.

To avoid non-amidine derivative formation (Browne & Kent, 1975) and still retain a certain degree of α-amino selectivity (Hunter & Ludwig, 1962), the acetimidation reaction was conducted in the pH range 9.5–11.0. The appearance of irreversible acetimidomyoglobin products (A_3, A_4, and A_5) was much in evidence even at a pH value of 9.5. However, their total production dropped drastically (~80%) with an increase of pH of just 1.5 units (Table III). Even in the extreme case represented by the first entry in Table III at most only 5% of the lysine residues are irreversibly modified.

Recently, there has been increased use of the acetimidyl group for semisynthetic purposes (Garner & Gurd, 1975; Gurd et al., 1977; Wang et al., 1977; Fölsch, 1975; Slotboom & de Haas, 1975; Harris & Offord, 1977) as a result of its hydrophilic nature and stability to extreme acidic conditions. Additionally, the alteration of product distribution with small changes in reaction conditions makes it an extremely versatile means of protection. However, deacetimidation conditions are quite strenuous (Ludwig & Byrne, 1962) and have been avoided for some purposes since acetimidoproteins exhibit close physical similarity with the unmodified protein (Slotboom & de Haas, 1975; Harris & Offord, 1977). Model studies on pentapeptides (McKerrow & Robinson, 1971; Scotchler & Robinson, 1974) suggest the possibility of partial deamidination, of asparagine in particular, under deactimidation conditions. No evidence of this was found as each of the six acetimidomyoglobins yielded only one electrophoretically homogeneous product after ammonolysis. Apparently the ammonium hydroxide–acetic acid buffer employed impedes the rate of deamidination in a similar manner as Tris buffer. Formation of irreversible by-products is a drawback of this method but their production can be limited. Obviously, all uses of the acetimidyl group in protein modification studies should be followed by appropriate purification and identification methods since formation of more than one derivative is highly probable.

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Supplementary Material Available

Experimental material including elution profiles, peptide compositions, and repetitive yield plots (16 pages). Ordering information is given on any current masthead page.

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Reduced Nicotinamide Adenine Dinucleotide-Cytochrome b_5 Reductase: Location of the Hydrophobic, Membrane-Binding Region at the Carboxyl-Terminal End and the Masked Amino Terminus†

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ABSTRACT: Microsomal NADH-cytochrome b_5 reductase is an amphiphilic protein consisting of a hydrophilic (catalytic) region and a hydrophobic (membrane-binding) segment. Digestion of the reductase purified from rabbit liver microsomes with carboxypeptidase Y (CPY), but not with aminopeptidases, resulted in the abolishment of the capacities of the reductase to bind to phosphatidylcholine liposomes and to reconstitute an active NADH-cytochrome *c* reductase system upon mixing with cytochrome b_5. The NADH-ferricyanide reductase activity of the flavoprotein was, however, inactivated only slightly by the CPY digestion. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and amino acid analyses indicated that the CPY treatment removed about 30 amino acid residues from the COOH terminus of the reductase and that about 70% of the amino acids released were hydrophobic. It is concluded that the hydrophobic region of the reductase, responsible for both membrane binding and effective reconstitution of NADH-cytochrome *c* reductase activity, is located at the COOH-terminal portion of the molecule. No NH_2-terminal residue could be detected in the intact and CPY-modified reductase preparations. The location of the hydrophobic, membrane-binding segment at the COOH-terminal end and the masked NH_2 terminus have also been reported for cytochrome b_5, another microsomal membrane protein.

The hepatic microsomal flavoprotein, NADH-cytochrome b_5 reductase, is an amphiphilic protein consisting of a hydrophilic moiety carrying the FAD prosthetic group and a hydrophobic segment (Spatz & Strittmatter, 1973; Mihara & Sato, 1975). It is composed of a single peptide chain having a molecular weight of about 33 000 and exists in aqueous solution as an oligomeric aggregate possessing an apparent molecular weight of about 360 000 (Mihara & Sato, 1975). The hydrophobic segment has been shown to be required for the tight binding of the reductase to various natural and artificial lipid bilayer membranes (Rogers & Strittmatter, 1974; Mihara & Sato, 1975) and for effective reconstitution of NADH-cytochrome *c* reductase activity upon mixing with the intact form of cytochrome b_5 (Mihara & Sato, 1972, 1975; Okuda et al., 1972). Digestion of liver microsomes with lysosomes (Takesue & Omura, 1970a) or a lysosomal acid protease (St. Louis et al., 1970) leads to solubilization of a hydrophilic, FAD-containing fragment of the reductase. This fragment has a molecular weight of about 27 000 and retains a high NADH-ferricyanide reductase activity (Takesue & Omura, 1970b). However, nothing is known of the location of the hydrophobic segment having the aforementioned properties in the structure of the reductase. In this paper, we report evidence obtained by the use of CPY[1] as modifying agent that the COOH-terminal portion of the reductase molecule constitutes the hydrophobic segment. We also report that the NH_2-terminal residue of the reductase is chemically masked. These observations are of special interest in view of the fact reported by Ozols (1974) that cytochrome b_5, another microsomal amphiphilic protein (Ito & Sato, 1968; Spatz & Strittmatter, 1971), also has its hydrophobic, membrane-binding segment at the COOH-terminal end and a masked NH_2-terminal residue.

Experimental Procedure

Materials. The intact forms of NADH-cytochrome b_5 reductase and cytochrome b_5 were purified from rabbit liver microsomes to homogeneity as described by Mihara & Sato (1975) and Spatz and Strittmatter (1973), respectively. The purified preparations were free from detergents and phospholipids. A hydrophilic, FAD-containing fragment of NADH-cytochrome b_5 reductase ("cathepsin-solubilized reductase") was purified also from rabbit liver microsomes as follows. Microsomes (36.7 g of protein) were digested with partially purified cathepsin D (1.96 g of protein) in 2.36 L of 0.1 M Tris–maleate buffer (pH 5.6) containing 1 mM EDTA at 37 °C for 3 h and then the mixture was centrifuged at 65 000*g* for 1 h. The reductase thus solubilized and recovered in the supernatant fluid was purified by ammonium sulfate fractionation, Sephadex G-100 gel chromatography, and DEAE-cellulose column chromatography as described by Takesue & Omura (1970b). The concentrated reductase preparation thus obtained (in 50 mM Tris-HCl buffer (pH 8.0) containing 50 mM KCl) was diluted 10-fold with distilled water and applied to a CM-Sephadex C-50 column equilibrated with 10 mM sodium phosphate buffer (pH 6.5). After

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[1] Abbreviations used are: CPY, carboxypeptidase Y; $NaDodSO_4$, sodium dodecyl sulfate; CD, circular dichroism.

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