SEMISYNTHESIS OF A SPECIFIC NH_2-TERMINAL [1-^{13}C] GLYCINE ADDUCT TO SPERM WHALE MYOGLOBIN: INTERMEDIATE PROTECTION OF ε-AMINO GROUPS WITH METHYL ACETIMIDATE

W. H. Garner and F. R. N. Gurd

Department of Chemistry
Indiana University
Bloomington, Indiana 47401

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Summary

The reaction of methyl acetimidate with sperm whale ferrimyoglobin at pH 10.5 is shown to lead to preferential coupling to the ε-amino groups forming the ε-acetimidolysine derivatives. The ε-acetimidolysine derivative removes the nucleophilicity of the ε-amino group so that subsequent coupling of tert-butyloxycarbonyl-[1-^{13}C] glycine-N-hydroxysuccinimide ester at pH 7.2 is directed to the NH_2-terminal valine residue. The deprotected specifically enriched N^{α}-[1-^{13}C] glycine adduct to the reconstituted, native ferrimyoglobin is determined by ^{13}C-NMR measurements to have a pK value of 7.8.

Semisynthesis of a protein or protein derivative by coupling natural or synthetic fragments (1-10) generally requires reversible introduction of protective groups into large peptides, except in certain special cases (9). As a step towards the general development of such a strategy we report here the use of methylacetimidate as a protective group to limit coupling through ε-amino groups during the addition of 71% ^{13}C enriched t-butyloxycarbonyl [1-^{13}C] glycine-N-hydroxysuccinimide ester to the α-amino terminus of sperm whale ferrimyoglobin. The use of the ^{13}C label contributes directly to the structure proof as well as illustrating the potentiality of specific chemical incorporation of an enriched residue for ^{13}C NMR studies (4, 6-8, 11).

Preferential modification of ε-amino groups by methylacetimidate was established by Hunter and Ludwig (12). The present report extends that procedure to include protection and deprotection of ε-amino groups with methylacetimidate so that it is compatible with the directed coupling of N-protected activated glycine to the α-amino terminus.

Materials and Methods

Preparation of N^{α} [1-^{13}C] Glycylmyoglobin. A sample of 600 mg of myoglobin was treated with 3.5 g methylacetimidate (12) for 2 hours at 16° at a constant pH 10.5 in a pH-stat. The modified protein was freed of small molecules on a G-10 Sephadex column (5 x 30 cm) at 16° equilibrated with 0.1 M Tris buffer at pH 10.0, followed by dialysis against deionized water. The poly-N^{ϵ}-acetimidyl myoglobin was treated with 150 mg of t-butyloxycarbonyl [1-^{13}C] glycine-N-hydroxysuccinimide ester (12, 14) at constant pH 7.1 at 16° for 4 hours. Following dialysis against deionized water, the acetimidyl groups were removed by adding a 4% solution of the protein to a concentrated ammonia-acetic acid mixture (15:1, v/v) at an apparent pH of 11.5 and 16° (15, 16). After ammonolysis for 9 hours[1] the solution was dialyzed to neutrality and reconcentrated to a 1% protein solution. The treated protein was freed of heme (17), dialyzed against water, and lyophilized. The t-butyloxycarbonyl group was removed in 5 ml of anhydrous trifluoroacetic acid containing 0.1 ml anisole and 0.1 g of dithioerythritol. After 10 minutes at room temperature the acid was removed by flash evaporation. The viscous apoprotein residue was rehydrated in 100 ml of O_2-free deionized water, and dialyzed to neutrality. The holoprotein was reconstituted with heme (18), and deionized. The enriched protein was purified by chromatography (19) and concentrated by ultrafiltration (Amicon Corp.).

Results and Discussion

Chemical Characterization. Results of amino acid analysis by the single column method (Durrum Chemical Corp.) following acid hydrolysis are shown in Table I. Residues per molecule for the acetimidated preparation are compared with the values for the untreated myoglobin. The resolution of ε-acetimidolysine after histidine on the single column methodology improves quantitation of the initial concentration[2] of the lysine derivative. Previous results had indicated extensive incorporation of glycine in the preparation without protection of ε-amino groups (6).

The upper section of Table II contains an end group analysis of the distribution of forms of α-amino group modification determined by automated Edman degradation with the Beckman Sequencer model 890C, carried through several rounds of cleavage. The adduct glycine, the normally terminal valine and the second residue leucine were quantitated by acid hydrolysis and amino acid analysis (20) in each of the successive rounds. At the same time an

[1] The $t_{\frac{1}{2}}$ for the removal of acetimidyl groups was later found to be 4 hours under these conditions, so that a period of 24 hours would be preferable. Such a length of exposure has proved harmless to the protein under these conditions.

[2] The pseudo first order rate constant of hydrolysis of ε-acetimidolysine in 6 N HCl at 110° was determined to be 0.5 x 10^{-2} hr^{-1}.

TABLE I

Composition of Semisynthetic Myoglobin

Preparations were analyzed by acid digestion in the usual manner with appropriate averaging and extrapolation of values measured with hydrolysis times of 24, 48 and 72 hours (13). The ε-acetimidolysine content is corrected for partial destruction during hydrolysis.

	Protein		
Amino Acid	Tert-butyloxycarbonyl [1-^{13}C] Glycine Acetimidated Myoglobin	Deprotected N^{α}[1-^{13}C] Glycylmyoglobin	Native Myoglobin
Aspartic Acid	8.2	8.3	8.3(8)[a]
Threonine	4.6	4.9	4.6(5)
Serine	5.7	5.8	5.3(6)
Glutamic Acid	19.1	19.5	19.0(19)
Proline	5.1	4.9	5.2(5)
Glycine	11.6	11.5	10.9(11)
Alanine	17.3	17.2	16.7(17)
Valine	7.3	7.7	7.4(8)
Methionine	2.0	2.1	2.1(2)
Isoleucine	7.9	8.1	8.6(9)
Leucine	17.7	18.0	18.0(18)
Tyrosine	3.3	3.1	2.9(3)
Phenylalanine	6.5	6.5	6.2(6)
ε-acetimidolysine	19.0	3.6	--
Lysine	--	13.7	19.5(19)
Histidine	12.5	12.5	12.6(12)
Arginine	4.3	3.8	4.1(4)

[a] The composition is given in parentheses according to A. B. Edmundson, Nature 205, 883-887, 1965

aliquot for total hydrolysis established the correct basis for computing fractional yields. The yield of valine in the second degradation cycle reflects the amount of N^{α}-glycine adduct originally present. That for the N^{α}-acetimidyl form was obtained by difference, as were the values for N^{ϵ}-[1-^{13}C] glycine. From these data the mole fraction of the adduct is shown

TABLE II

End Group and Physical Parameters of Semisynthetic Myoglobin

The amino adducts were determined by the automated Edman degradation method (21). Parameters compared are the ratio of molar extinction coefficient at 409 and 280 nm, the molar ellipticity at 208 nm, determined respectively from measurements of optical absorbance and circular dichroism.

Amino Adducts	Tert-butyloxycarbonyl [1-^{13}C] Glycine Acetimidated Myoglobin	Deprotected N^{α}[1-^{13}C] Glycylmyoglobin	Native Myoglobin
N^{ϵ}-[1-^{13}C] glycine	0.0	0.1	
N^{ϵ}-acetimidyl	19.0	3.6	
N^{α}-[1-^{13}C] glycine	0.3	0.4	
N^{α}-acetimidyl	0.6	0.2	
Total unmodified N^{α} protein	0.0	0.3	1.0
Parameter			
$\epsilon_{409}:\epsilon_{280}$	5.0	5.4	5.36
$[\theta]_{208}$, deg cm^2/decimole	-23700	-24400	-23400

in Table II (21). The results in Table II show that the yield of N^{α}-[1-^{13}C] glycyl derivative involves 0.4 to 0.5 residues per molecule, and that with inclusion of the methylacetimidate modification step the degree of glycine coupling to ε-amino groups is very slight.

The physical parameter values as shown in the lower section of Table II for the absorbance ratio at 409 to 280 nm (Cary model 14) and for the mean residue ellipticity, $[\theta]_{208}$ (Jasco-Sproul Scientific SS-10 CD modification) reflect the properties of the native protein. Separate preparations in which ammonolysis was complete[1] yielded corresponding preparations with respect to α-amino modification and physical properties.

The heterogeneity with respect to the amino terminus does not detract

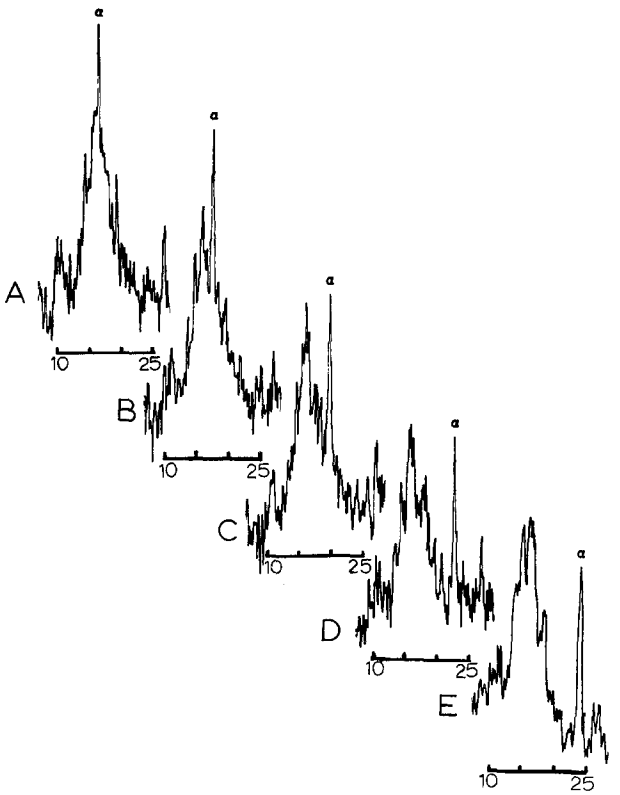

Figure 1. ^{13}C Fourier transform nuclear magnetic resonance spectra of N^{α}-[1-^{13}C] glycylmyoglobin at various pH values: A, pH 9.27; B, pH 8.82; C, pH 7.99; D, pH 7.11; E, pH 6.48. The single adduct peak is marked α. Proton-decoupled spectra were accumulated 16,384 times at 32 ± 1°. Recycle time was 1.6 s. Protein concentration was 3.2 mM. Chemical shifts are expressed upfield of external CS_2, with internal dioxane standards in every case taken as 126.2 ppm.

from the NMR measurements on the enriched adduct. However certain chemical strategies can be employed if necessary to separate the free α-amino terminal species from the acetimidated α-amine species prior to coupling the N-protected activated amino acid; these include modification of the amino function with citraconic anhydride followed by ion exchange chromatography and isolation of the appropriate chemical species followed by removal of the citraconyl group (22).

Characterization by ^{13}C NMR. The appropriate region of the ^{13}C NMR spectrum is shown in Fig. 1. Five parts, A through E, are shown progressing

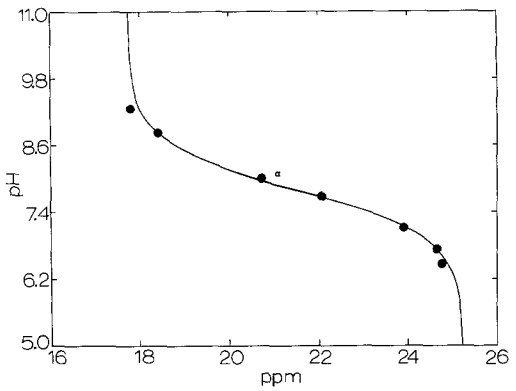

Figure 2. Dependence on pH of the chemical shift of the adduct resonance shown in Figure 1. Chemical shift values were taken from computer channel positions. The curve is theoretical according to the Henderson-Hasselbalch equation.

through decreasing ranges of pH. The single α-amino adduct resonance is marked in each part by the symbol α. The pH dependence of the chemical shift of this resonance is shown in Fig. 2, fitted by a pK value of 7.80. The pK value found for the virgin protein is 7.79 (23, 24). The chemical shift range observed is in keeping with model peptide studies (25).

The synthetic procedure outlined here is being adapted for the preparation of a des-1 protein and to the coupling of L-valine in order to extend the method for the direct study of interactions of NH_2-terminal residues in myoglobins and hemoglobins.

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