Carbon 13 Nuclear Magnetic Resonance Spectroscopy of Myoglobins Carboxymethylated with Enriched [2-13C]Bromoacetate*

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SUMMARY

Cyanoferrimyoglobins of harbor seal and sperm whale were carboxymethylated with enriched [2-13C] bromoacetate. The enriched adducts were readily observed by 13C nuclear magnetic resonance. Resonances were identified by comparison with enriched adducts to appropriate small molecules, and T1 values were determined under various conditions. In addition to the expected adducts to the NH2-terminal residues and to histidine and lysine, an alkali-labile glycolate ester product was observed. Denatured forms were obtained by carboxymethylation in 8 M urea which led to complete conversion of all histidine residues to the di-carboxymethyl form. By carrying out carboxymethylation in stages it was possible to limit the labeling to either the normally external or the normally internal set of histidine residues. Treatment under denaturing conditions led to carboxymethylation of both methionine residues. The T1 values place some limits on the interpretation of the degrees of mobility of the adduct carbon nuclei.

The immediately preceding reports (1, 2) provide a detailed basis for the description of the results of 13C nuclear magnetic resonance studies of myoglobins carboxymethylated with enriched [2-13C] bromoacetate (3). The methylene carbons of the carboxymethyl adducts are readily visualized in the protein spectra, and relaxation measurements can be followed over an appreciable range of concentration. Minor products that are easily overlooked in chemical analysis are detectable. Model studies have been made to facilitate assignment of all resonances attributable to the enriched carbon nuclei. The results indicate the potential of protein enrichment for 13C nuclear magnetic resonance techniques (4-9).

EXPERIMENTAL PROCEDURE

Protein Modification—The carboxymethylation and analytical procedures have been described (1, 2). The NH2-terminal analysis was according to Stark and Smyth (10). Concentration procedures and conversion to the cyanoferrimyoglobin derivatives were achieved as described previously (1).

Peptide Modification—The pentapeptides, glycylglycyl-n-L-lysylglycylglycine and glycylglycyl-L-methionylglycylglycine were prepared as part of another study (11). α-N-Acetyl-L-histidine was obtained from Calbiochem. L-Valyl-L-leucyl-L-seryl-n-glutamylglycine was a preparation used previously (12). To determine the chemical shifts of the NH2-terminal derivatives, the lysine-containing pentapeptide, 81 nmoles dissolved in 3 ml of deionized water was treated with a 4:1 mole ratio of 90% enriched [2-13C] bromoacetate at pH 7.6. The reaction was performed under a nitrogen atmosphere and controlled by the pH stat at 25° (2). After 12 hours the reaction was stopped by titrating the solution to pH 1.8. The excess bromoacetate was removed by three extractions with equal volumes of ether. The aqueous layer was taken to dryness, dissolved in 2 ml of water, and adjusted to pH 5.96. The sample, following the addition of one drop of dioxane as an internal standard, was analyzed by the NMR technique (1). Following that, the reaction was continued at pH 10.5 to obtain an ε-amino derivative and worked up as before for the NMR analysis. Another sample of the pentapeptide was treated with the bromoacetate at pH 9.5 for 24 hours, 40 nmoles, 4:1 molar ratio of bromoacetate, in 3 ml of 0.1 M borate buffer.

The pentapeptide with NH2-terminal valine was carboxymethylated at pH 7.5 under the same conditions as described above for the lysine-containing pentapeptide. Derivatives of histidine were obtained by treatment of α-N-acetyl-L-histidine (160 nmoles) with a 2:1 molar ratio of 75% enriched [2-13C] bromoacetate in 3 ml of 0.5 M phosphate buffer, pH 6.8, for 25 hours. The reaction was stopped as described above.

The carboxymethionine derivative was prepared by

treated the methionine-containing pentapeptide with a 4:1 molar proportion of 75% enriched [2-13C]bromoacetate at pH 5.5 in 0.5 M phosphate for 24 hours.

**Synthesis of Enriched [2,13C]Bromoacetate**—The procedure was modified from that of Shaw (13). One gram of 60% enriched [2,13C]acetic acid (Mallinckrodt Chemical Works) was pipetted into a 5-ml round bottom flask, containing a magnetic stirring bar, and fitted with a side arm. Following addition of 0.037 ml of acetic anhydride a reflux condenser and cold finger assembly fitted with a drying tube were placed above the flask. Through the side arm 0.1 to 0.2 ml of dry and distilled bromine was added. After heating the mixture for about 30 min in an oil bath at 90° the remainder of the stoichiometric amount of bromine was added, and the reaction allowed to proceed for a few hours. When the deep red color had lightened, a further 0.2 to 0.3 ml of bromine was added and the reaction maintained for 2 more hours. Finally, excess bromine was carried off in a stream of dry air. The remaining solid was recrystallized from anhydrous ethyl ether and petroleum ether at 4°. Yields were normally between 70 and 80%. The melting point was 49 to 50°.

C₇H₅BrO₂
Calculated: C 17.29, H 2.17, Br 57.51, O 23.02
Found: C 16.02, H 2.45, Br 57.24, O 22.82

Mass spectrometry showed the enrichment with respect to 13C to be 59%. A similar preparation from nominally 90% enriched [2,13C]acetic acid (IsoMet) yielded a confirmed 75% 13C enrichment in the bromoacetic acid.

**Synthesis of γ-Carboxymethyl Ester of L-Glutamic Acid**—The procedure of Takahashi et al. (14) yielded a product heavily contaminated with starting materials that was partially purified by Dowex 50-X8 chromatography on a column (5 × 27 cm) developed with 0.2 M pyridine acetate buffer at pH 3.1. The product finally used for the NMR studies was still contaminated with glycic acid. The elution profile on the amino acid analyzer corresponded to that obtained by Takahashi et al. (14).

**RESULTS AND DISCUSSION**

**Carboxymethyl Derivative of Harbor Seal Cyanoferrimyoglobin**—Harbor seal ferrimyoglobin was treated in the usual manner (2) for 6 days, with 30% enriched [2-13C]bromoacetate. The composition was very close to that given previously (Ref. 2, Table 1). The pertinent values, expressed as residues per molecule, are 6.5, 17, 1.2, and 0.7 for histidine, dicarboxymethylhistidine, e-carboxymethylhistidine, and δ-carboxymethylhistidine, respectively, as well as 11.1 and 18.4 for glycine and lysine, respectively. The NH₂-terminal determination of Stark and Smyth (10) gave a value of 0.1 residue of glycine, indicating extensive modification of this residue.

The sample was concentrated in the Amicon apparatus to 14 mm, made 0.1 M in phosphate, pH 7.0, and converted to the cyan compound form (1). Fig. 1 shows a comparison of the pulsed Fourier transform 13C NMR spectra near pH 7 of unmodified myoglobin (Fig. 1A), harbor seal myoglobin treated with bromoacetate, at natural abundance with respect to isotopes (1) (Fig. 1B), and the harbor seal myoglobin treated as described above (Fig. 1C). In Fig. 1C four resonances stand out from the protein background, namely at 129.4, 134.4, 141.0, and 143.3 ppm upfield from CS2. The C1 resonance of lysine at 153.4 ppm (7) was barely recognizable.

**Fig. 1.** Proton-decoupled Fourier transform 13C NMR spectra of harbor seal cyanoferrimyoglobin in 0.1 M phosphate. Resonance positions are given in parts per million upfield from CS₂. A, untreated myoglobin, pH 7.41, 14 mM, 16,384 accumulations, 1.360 s recycle time, 25°; B, myoglobin treated with bromoacetate at natural abundance with respect to isotopes (1), pH 7.08, 12 mM, 60,000 accumulations, 1.360 s recycle time, 25°; C, myoglobin treated as described for B but with 30% enrichment of the methylene carbon of the bromoacetate (see text), pH 7.27, 14.5 mM, 8,192 accumulations, 0.555 s recycle time, 20°.

**Carboxymethyl Derivative of Sperm Whale Cyanoferrimyoglobin**—Sperm whale ferrimyoglobin was treated in the same manner with 60% enriched [2,13C]bromoacetate for 8 days. The composition again was very close to that given previously (Ref. 2, Table 1). The pertinent values, expressed as residues per molecule, are 6.0, 3.9, 1.5, and 0.8 for histidine, dicarboxymethylhistidine, e-carboxymethylhistidine and δ-carboxymethylhistidine.

Simply from the standpoint of their size, the large twin peaks in Fig. 1C would be expected to contain the majority of the resonances of the histidine derivatives, with each of the resonance positions being one of the two possible adducts on the imidazole ring. Close scrutiny of Fig. 1B shows that the twin resonances make their appearance on the upfield edge of the α carbon envelope. An expanded view of the region of interest, accumulated over a 50-ppm window is shown in Fig. 2. This spectrum confirms the presence of only four adduct bands and brings out more clearly the greater width of the resonance band centered at 143.3 ppm upfield from CS2. The protein background is barely recognizable.

**Fig. 2.** Expanded view of the region of the resonances of the enriched carbon sites in Fig. 1C, accumulated over a 50-ppm window. The conditions were unchanged except that 6640 accumulations were obtained at a recycle time of 3.100 s.
the corresponding monocarboxymethylglycine derivative. By amino acid analysis it was found to contain monocarboxymethylvaline. This result matches the chemical shift of available (12). The peptide yielded on carboxymethylation a product that had an additional resonance (12) at 143.0 ppm at field peaks were taken as the dicarboxymethyl and monocarboxymethyl derivatives, respectively.

The region of the 13C NMR spectrum corresponding to that occupied by the adducts is shown in A, where the sulffhydryl of CS2 is absent from the spectrum of the sperm whale adduct. Various peptide derivatives were then studied to permit identification of the adduct resonances.

**Peptide Derivatives: NH2-terminal**—The sample of glycylglycyl-L-methionyl-valine following the modification. In keeping with previous experience (15), a low recovery of about 70% was obtained with the untreated sperm whale myoglobin.

The region of the 13C NMR spectrum corresponding to that occupied by the adducts is shown in A, where the sulfhydryl of CS2 is absent from the spectrum of the sperm whale adduct. Various peptide derivatives were then studied to permit identification of the adduct resonances.

**Peptide Derivatives: NHz-terminal**—The sample of alanine treated as described above at pH 10.5 showed a resonance at 135.6 ppm at pH 7.32 ascribable to the dicarboxymethyl derivative of the e-amino group of the lysine residue, in addition to the dicarboxymethylglycine resonance. The presence of this lysine derivative but not of the mono derivative was confirmed by amino acid analysis. A separate treatment at pH 9.5 for 24 hours produced both e-amino derivatives in addition to the monocarboxymethylglycine. Spectral measurements at pH values of 7.32, 8.34, and 10.35 made it possible to recognize the resonance of the monocarboxymethyllysine adduct, which appeared at 143.5, 143.4, and 141.4 ppm under these three conditions, respectively.

**Peptide Derivatives: Histidine Imidazole Group**—The reaction product obtained from the treatment of α-N-acetyl-L-histidine was found on amino acid analysis to contain the e-carboxymethyl, δ-carboxymethyl, and δ,ε-dicarboxymethyl derivatives of histidine in the molar ratio of 3:1:1. The 13C NMR analysis at pH 7.02 gave chemical shifts of 140.8 and 143.1 ppm which could be recognized from separate experiments as representing the e- and δ-adducts in the dicarboxymethyl derivative. The most intense resonance, at 142.9 ppm, could be ascribed directly to the e-carboxymethyl adduct in the monoadduct derivative. The resonance at 144.0 ppm was taken to represent the δ-carboxymethyl adduct in the δ-monocarboxymethyl derivative. The areas under the assigned peaks corresponded to the ratios expected from amino acid analysis. This sample was then treated further with a 15-fold molar ratio of unenriched bromoacetate at pH 7.2 for 36 hours. The most intense peak, still representing the e-adduct but now contained in the dicarboxymethylhistidine form, was shifted from 142.2 ppm to 140.8 ppm at pH 7.02. Similarly, the original resonance at 144.0 ppm moved coincident with the resonance at 143.1 ppm, still representing the δ-adduct but now contained in the dicarboxymethylhistidine form.

**Peptide Derivative: Methionine Thiocysteine Group**—The methionine sulfoxonium salt formed by carboxymethylation with the [2-13C]bromoacetate of the pentapeptide glycylglycyl-L-methionyl-glycine showed a single strong resonance at 145.7 ppm at pH 6.21.

Table I summarizes the chemical shift positions for the various carboxymethylated derivatives dealt with above.

**Assignment of Recognized Derivatives in Protein Spectra**—The resonances in Fig. 1C or Fig. 2 at 134.4, 141.0, and 143.3 ppm can be assigned dominantly to the dicarboxymethyl derivative of glycine, the e-carboxymethyl adduct in dicarboxymethylhistidine, and the δ-carboxymethyl adduct in dicarboxymethylhistidine, respectively. The presence of monocarboxymethyl derivatives of the terminal glycine or lysine side chains, as well as either of the monocarboxymethyl derivatives of histidine

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1. The formation of this derivative was postulated previously on tentative grounds (17).
The amino acid residues listed were alkylated with [2-13C]bromacetate to obtain enriched carboxymethyl adducts whose characteristic resonance positions are expressed as parts per million upfield of CS2. Reaction conditions are given in the text. The 13C NMR measurements were made near 28°C. The four sets of observations were made at pH 7.02, 7.52, 7.52, and 6.21, respectively.

<table>
<thead>
<tr>
<th>Residue carboxymethylated</th>
<th>Derivative observed</th>
<th>Chemical shift</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histidine</td>
<td>δ-Monocarboxymethyl</td>
<td>144.0</td>
</tr>
<tr>
<td></td>
<td>ε-Monocarboxymethyl</td>
<td>142.2</td>
</tr>
<tr>
<td></td>
<td>Dicarboxymethyl individual adducts</td>
<td>143.1</td>
</tr>
<tr>
<td></td>
<td>ε-Carboxymethyl</td>
<td>140.8</td>
</tr>
<tr>
<td>Glycine</td>
<td>NH2-terminal adducts</td>
<td>142.0</td>
</tr>
<tr>
<td></td>
<td>Monocarboxymethyl</td>
<td>134.0</td>
</tr>
<tr>
<td></td>
<td>Dicarboxymethyl</td>
<td>135.6</td>
</tr>
<tr>
<td>Lysine</td>
<td>ε-NH2 adducts</td>
<td>143.5</td>
</tr>
<tr>
<td></td>
<td>Monocarboxymethyl</td>
<td>134.0</td>
</tr>
<tr>
<td></td>
<td>Dicarboxymethyl</td>
<td>135.6</td>
</tr>
<tr>
<td>Methionine</td>
<td>Carboxymethylsulfonium salt</td>
<td>145.7</td>
</tr>
</tbody>
</table>

a Initially α N acetyl L histidine.
b Initially glycyglycyl-L-lysylglycylglycine.
c Initially glycyglycyl-L-methionylglycylglycine.

The results point to the ester adduct reported by Takahashi et al. (14) who observed the formation of the glycolate ester of a glutamic acid residue in carboxymethylated ribonuclease T1, the same type of reaction has been postulated in other cases (18, 19). The enriched methylene should have a chemical shift close to that observed (20, 21). As a free small molecule glycolate should undergo very rapid tumbling with a greatly lengthened relaxation time and the observed resonance should exhibit marked attenuation at the recycle time employed here (22). The γ-carboxymethyl ester of L-glutamic acid was then prepared (14). Assignments could be made for each of the carbon nuclei in the molecule, with the alcohol methylene located at 129.4 ppm, matching the position obtained in the protein spectra. The presence of free glycolate in the base-treated sample also allowed confirmation of the resonance at 131 ppm as C3 of glycolate, in keeping with a separate observation on an authentic glycolate sample.

The glycolate adduct remained with the polypeptide moiety during the short exposure to low pH as the heme was removed (23) from the carboxymethyl seal myoglobin. In an experiment to be described more fully later an unenriched carboxymethyl seal apomyoglobin preparation was further treated with [2-13C]bromacetate in the presence of urea (1). This experiment showed an intensity of the resonance at 129.4 ppm corresponding to the addition of approximately two glycolate ester groups coming on top of the one to two groups added in the first alkylation stage. Furthermore, this second stage was carried out with a disrupted protein structure. Most probably the glycolate ester formation depends on no special aid from neighboring residues, in contrast to the relatively facilitated reaction described for ribonuclease T1 (14). In the present case a discrimi-
adducts (141 and 143 ppm) are similar to those of the NH₂-terminal adduct (134 ppm) which is observable only in the seal protein derivative. The glycolate ester methylene (129 ppm) has nearly twice as large a \( T_1 \) value, reflecting more freedom of motion in this adduct attached to a relatively flexible side chain (1, 7, 11, 22, 26–28). The value for the NH₂-terminal adduct represents the average of the two carbon nuclei in this dicarboxymethyl derivative. Those for each of the histidine adducts represent averages over larger numbers of nuclei, about 6 or 7 modified residues. Even so, the relaxation of these adducts is characterized adequately for each resonance by a single \( T_1 \) value. Furthermore, the standard deviations of the \( T_1 \) values are of modest degree and no multiple components are observed in the relaxation spectra obtained near the null point.

As a first approximation each directly bonded hydrogen borne by a particular carbon will affect the relaxation of the carbon nucleus to the same degree, and hence comparison between carbon types can be made in terms of \( NT_1 \), where \( N \) is the number of such directly bonded hydrogens (1, 22, 27). The \( NT_1 \) value for the histidine and NH₂-terminal adducts in most cases is about 76 ms compared to 28 ms for the \( \alpha \) carbons of the protein (1). This result indicates that the enriched loci reflect some contributions from internal motion relative to the average motion of the \( \alpha \) carbons (7, 26–28). The \( NT_1 \) values found here could be classified in the range of those side chain carbons to which relatively low mobility has been ascribed (7). Further comparisons are postponed until the results on the denatured conformations have been presented.

### Carboxymethylated Apomyoglobin Spectra

Before turning to some enriched preparations corresponding to the highly carboxymethylated preparation that was studied in great detail in the preceding paper (1) to show the behavior of a denatured myoglobin, it is instructive to observe the effects of pH on the spectrum of the apomyoglobin prepared from a normally alkylated sample of the seal protein. The sample treated for 6 days with 30% enriched [2-\( ^{13} \)C]bromacetate, shown in Fig. 1C and presented in the first two entries in Table II, was freed of heme and studied under different conditions of pH. Apomyoglobin is known to undergo conformational changes at high pH (23), and carboxymethylated myoglobins experience conformational changes near pH 5 and pH 11 (29).

The spectra in Fig. 5 show the carboxymethyl cyanoferri myoglobin from harbor seal from which the apomyoglobin was prepared (Fig. 5A), to be studied at pH 5.00 (Fig. 5B), pH 9.39 (Fig. 5C), and pH 12.24 (Fig. 5D). The spectrum at pH 5.00 deserves attention representing as it does a pH region where aggregation of the protein is incipient but structural disruption is not yet observed (23, 29). The effects seen in Fig. 5B include line broadening and reduction of intensity, both apparent throughout the spectrum (B) compared to A or C, but most clearly seen with the major adduct resonances. The area under these resonances is in fact reduced to about one-half of its value in the unnaggregated preparations. It is possible that aggregation has reached the point where signal strength is much attenuated. The decrease in signal intensity may also reflect the effects of an increased \( T_2 \) on \( T_1 \), reflected in line width, and on the nuclear Overhauser enhancement, reflected in intensity of these proton-decoupled spectra (27). Such an argument puts certain limits on the value of the correlation times describing the motion of the enriched adducts (27). Furthermore, the possibility of exchange broadening cannot be ignored. If such an effect contributes to spin-lattice relaxation it might also reduce the nuclear Overhauser enhancement.

### Table II

<table>
<thead>
<tr>
<th>Enrichment (%)</th>
<th>Sample conditions</th>
<th>Chemical shift of resonance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH</td>
<td>Concentration (mM)</td>
</tr>
<tr>
<td>Carboxymethylated seal cyanoferrimyoglobin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>7.27</td>
<td>14.0</td>
</tr>
<tr>
<td>30</td>
<td>7.06</td>
<td>4.5</td>
</tr>
<tr>
<td>75</td>
<td>6.87</td>
<td>0.9</td>
</tr>
<tr>
<td>Carboxymethylated sperm whale cyanoferrimyoglobin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>6.94</td>
<td>4.0</td>
</tr>
<tr>
<td>60</td>
<td>6.88</td>
<td>2.0</td>
</tr>
</tbody>
</table>

An important advantage of the \( ^{13} \)C enrichment is the increased spectral sensitivity gained. This has made possible the study of the four prominent resonances, identified by chemical shifts expressed in parts per million upfield of C₆. The cyanoferri myoglobin derivatives are grouped according to animal species, and the bromoacetate percentage enrichment used, pH, and concentration of measurement are listed. Methods of preparation and \( T_1 \) determination are referred to in the text. The probe temperatures were stable within 1° during each experiment. Dilutions were with 0.1 M phosphate buffer, pH 6.8. Computed standard deviations are given in parentheses. The average signal-to-noise ratio for the major resonances was 8:1.

Relaxation Behavior of Adducts in Native Proteins—Table II lists the values of the spin-lattice relaxation time, \( T_1 \), in milliseconds, for various concentrations of one carboxymethyl derivative of the harbor seal and sperm whale cyanoferri myoglobins. The resonances of each enriched adduct are represented, with standard deviations given in parentheses. With the exception of the resonance at 134 ppm, which is absent from the sperm whale myoglobin spectrum (Fig. 3B), the patterns are very similar between the two species. This is in agreement with the numerous points of similarity seen in the natural abundance spectra (1) and the general similarity of the histidine reaction patterns (2). Considering the possibilities for heterogeneous distribution of sites of glycolate ester formation it is not surprising that some of the standard deviations are quite large for \( T_1 \) of the glycolate resonance at 129 ppm. The relatively low signal-to-noise for this resonance also contributes to the magnitude of the standard deviations observed.

An important advantage of the \( ^{13} \)C enrichment is the increased spectral sensitivity gained. This has made possible the study of a 15-fold concentration range covered in Table II. The absence of clear trends in \( T_1 \) with concentration, as well as of any indications of line broadening or discontinuities in relative signal-to-noise due to aggregation (24), is evidence that the high concentrations required for the NMR do not bring with them intractable intermolecular interactions. The self-diffusion study of myoglobin by Riveros-Moreno and Wittenberg (25) showed that below about 6 mM very little concentration dependence was evident in translational diffusion.

The relaxation times for the methylene carbons of the histidine adducts (141 and 143 ppm) are similar to those of the NH₂-terminal adducts (134 ppm) which is observable only in the seal protein derivative. The glycolate ester methylene (129 ppm) has nearly twice as large a \( T_1 \) value, reflecting more freedom of motion in this adduct attached to a relatively flexible side chain (1, 7, 11, 22, 26–28). The value for the NH₂-terminal adduct represents the average of the two carbon nuclei in this dicarboxymethyl derivative. Those for each of the histidine adducts represent averages over larger numbers of nuclei, about 6 or 7 modified residues. Even so, the relaxation of these adducts is characterized adequately for each resonance by a single \( T_1 \) value. Furthermore, the standard deviations of the \( T_1 \) values are of modest degree and no multiple components are observed in the relaxation spectra obtained near the null point.

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FIG. 5. Effect of pH on spectra of carboxymethyl apomyoglobins. A, control spectrum of carboxymethyl cyanoferrimyoglobin, as in Fig. 1C. This material was used as described in the text to prepare the apomyoglobin studied in Spectra B, C, and D. B, the apomyoglobin from A, pH 5.00, 5 mM, 20,624 accumulations, 0.555 s recycle time, 27°; C, same as in B, pH 9.39, 20,001 accumulations; D, same as in B, pH 12.24, 16,384 accumulations; E, a separate preparation of apomyoglobin of harbor seal obtained by first treating with 75% enriched [2-13C]bromoacetate for 6 days as the native protein, then removing the heme and treating the apomyoglobin in 8 M urea with unenriched bromoacetate as described in the text. The observations were at pH 6.86, 2 mM, 16,384 accumulations, 0.555 s recycle time, 32°.

Note that the spectrum in Fig. 5C at pH 9.39, 5.0 mM, resembles quite closely that in Fig. 5A for the ferrimyoglobin. Structural changes on removal of the heme are not very striking on a time-average observational basis (23, 30, 31). The similarities extend to line widths, intensities, and, as shown below, T1 values. However, in Fig. 5D, at pH 12.24, the spectrum becomes characteristic of a substantially altered state. There is an obvious narrowing of the resonance peaks, particularly of the adducts. The sharpening of the resonance at 134.4 ppm is very dramatic indeed. So far as the protein background is concerned in Fig. 5D, many examples of sharpening may be seen, not least in the carbonyl region. The spectrum is reminiscent of that of the fully carboxymethylated derivative studied previously at natural abundance (1). The conversion back from pH 12.24 to the pH 9.39 range was found to yield the spectrum in Fig. 5C in a fully reversible fashion. The areas under the adduct resonances were comparable in the two spectra, confirming that the nuclear Overhauser enhancement was probably near its maximum in the native structure (Ref. 27, Fig. 4).

Denatured Carboxymethylated Apomyoglobin Spectra Two preparations of harbor seal apomyoglobin were obtained in which all histidine residues were converted to the dicarboxymethyl form. In both cases a first treatment was made for 6 days under the standard alkylating conditions, followed by removal of the heme and then by a second, separate alkylation treatment in the presence of 8 M urea as described previously (1). In both cases the final product corresponded to a preparation already reported (1) and was found on analysis to contain no unmodified histidine or methionine and to have suffered a loss of 2 or 3 lysine residues. The two preparations were treated with 75% enriched [2-13C]bromoacetate in one stage and with natural abundance bromoacetate in the other. They differed in the essential way that the first was enriched in the first stage and the second in the second stage. The reaction patterns were very similar. The initial product contained 5.8 and 5.7 unmodified histidine residues per molecule in the two cases, respectively.

The preparation enriched in the first stage corresponds in its enrichment pattern to those groups modified in the native structure and is suitably included in Fig. 5 as the spectrum marked E. The comparison with Fig. 5A, C, or D shows first a comparable area under the main resonance bands in each case when allowance is made for differences in concentration, number of accumulations and vertical display scale. This observation is added support for the contention that the nuclear Overhauser enhancement is near maximum in all these cases (27). As expected for the disrupted structure obtained with the second alkylation stage (1) the spectrum in Fig. 5E is sharpened with relatively narrow resonance bands.

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The preparation enriched in the first stage corresponds in its enrichment pattern to those groups modified in the native structure and is suitably included in Fig. 5 as the spectrum marked E. The comparison with Fig. 5A, C, or D shows first a comparable area under the main resonance bands in each case when allowance is made for differences in concentration, number of accumulations and vertical display scale. This observation is added support for the contention that the nuclear Overhauser enhancement is near maximum in all these cases (27). As expected for the disrupted structure obtained with the second alkylation stage (1) the spectrum in Fig. 5E is sharpened with relatively narrow resonance bands.

FIG. 6. Comparison of spectra of harbor seal apomyoglobin carboxymethylated in two stages, one with enrichment at the native stage and the other with enrichment at the denatured stage. A, same as Fig. 5E, with enrichment at the native stage; B, the 75% enriched [2-13C]bromoacetate was applied at the stage of treatment of the apoprotein in 8 M urea. The pH values were 6.86 and 6.84, respectively. Except that the second spectrum is displayed at a higher vertical magnification, the conditions were identical: 2 mM, 16,384 accumulations, 0.555 s recycle time, 32°. Spectrum A is labeled with respect to adducts to the normally reactive sites in the native protein structure and Spectrum B with respect to those normally internally masked.

Enclosed diagram: Figure 6 shows the comparison of spectra of harbor seal apomyoglobin carboxymethylated in two stages, one with enrichment at the native stage and the other with enrichment at the denatured stage. A, same as Fig. 5E, with enrichment at the native stage; B, the 75% enriched [2-13C]bromoacetate was applied at the stage of treatment of the apoprotein in 8 M urea. The pH values were 6.86 and 6.84, respectively. Except that the second spectrum is displayed at a higher vertical magnification, the conditions were identical: 2 mM, 16,384 accumulations, 0.555 s recycle time, 32°. Spectrum A is labeled with respect to adducts to the normally reactive sites in the native protein structure and Spectrum B with respect to those normally internally masked.
The $T_1$ values are in milliseconds, and chemical shift positions are upfield of CS$_2$. Standard deviations are given in parentheses. Methods of preparation are given in the text. See Table II for further information. The signal-to-noise ratio for the last two preparations listed was at least 35:1. Otherwise it was 8:1 (Table II) or better.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sample conditions</th>
<th>Chemical shift of resonance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native, heme intact</td>
<td>pH 7.27, Concentration 14.0, Temperature 26.0°C</td>
<td>129 ppm 72(8), 134 ppm 40(1), 141 ppm 38(3), 143 ppm 38(3)</td>
</tr>
<tr>
<td>As above, heme removed</td>
<td>pH 9.39, Concentration 5.0, Temperature 26.5</td>
<td>129(16) 62(6), 134 ppm 47(7), 141 ppm 47(7)</td>
</tr>
<tr>
<td>Sample from pH 9.39</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Two stage, “external” label</td>
<td>pH 6.36, Concentration 2.0, Temperature 33.0</td>
<td>129 ppm 87(2), 141 ppm 89(2)</td>
</tr>
<tr>
<td>Two stage, “internal” label</td>
<td>pH 6.36, Concentration 2.0, Temperature 33.0</td>
<td>129 ppm 188(5), 141 ppm 171(0)</td>
</tr>
</tbody>
</table>

Normally internal nucleophilic groups became emphasized by alkylolation with the enriched [2,13C$_2$]branched ester. Two new resonances are present in Fig. 6B at 115.5 and 115.9 ppm. On the basis of the peptide model reactions already presented, these can be recognized as the methylene adduct carbons of the methionine carboxymethylsulfonium salt and some dicarboxymethyllysinine, respectively. No further formation of the terminal adduct, dicarboxymethylglycine, is indicated. As discussed earlier, the glycinate ester continued to be formed. The intensity of the resonance of the methionine adduct, representing two carbon nuclei per molecule, is of the expected relative magnitude. The contrasting results for methionine labeling in the two parts of Fig. 6 point up the specificity of the alkylolation reaction with respect to the intact protein structure more clearly than any other single piece of evidence obtained with myoglobin.

**Relaxation Behavior of Carboxymethyl Apomyoglobin**—The spin-lattice relaxation times, $T_1$, of the various enriched, carboxymethylated apomyoglobins are collected in Table III. The values of $T_1$ in milliseconds are given for the five resonances discussed above in terms of the carboxymethyl combination with the carboxyl groups (129 ppm), the amino terminus (134 ppm), the histidine $\epsilon$-derivative of the dicarboxymethyl form (141 ppm), both histidine monocarboxymethyl derivatives and the $\delta$-derivative of the dicarboxymethyl form (143 ppm), and the methionine side chains (146 ppm). The values already presented in Table II for the carboxymethyl cyanoferrimyoglobin of the harbor seal are entered first for reference, followed by the corresponding apomyoglobin form at pH 9.39 and pH 12.24. Then the values for the highly carboxymethylated denatured apomyoglobin preparations are presented, first with the labeling in the initial (native) stage and second with the labeling in the second (denatured) stage. For convenient reference, the normal Fourier transform spectra of these preparations are found, respectively, in Fig. 1C (also Fig. 2 and Fig. 3A), Fig. 5C, Fig. 5D, Fig. 6A (also Fig. 5E), and, finally Fig. 6B.

As shown in Table III, the removal of the heme had little effect on the major histidine adduct $T_1$ values. The low signal strength made necessary the omission of a value for the NH$_2$-terminal adduct in some cases. Slow loss of the glycinate ester peak likewise eliminated any reliable $T_1$ value for the 129-ppm resonance in alkaline solution. The material observed at pH 12.24 showed a significant increase in $T_1$ for the NH$_2$-terminal adduct and possibly significant increases for the histidine adducts. The latter resonances exhibited considerable heterogeneity in relaxation behavior under these conditions. The highly carboxymethylated, disrupted structure labeled in the first reaction stage, and therefore identical in enrichment pattern to the preparations already listed, showed very substantially longer $T_1$ values. Lastly, the preparation labeled in the second stage showed at least as long $T_1$ values for the histidine adducts as its converse preparation, as well as a longer value for the glycinate ester. The rather long $T_1$ value for the methionine adducts is noteworthy but not subject to comparison within this study.

In these last two preparations the histidine adducts are present solely in the dicarboxymethyl form. Hence, with the exception of a small overlap of resonance in the 143 ppm position of lysine adduct, the two peaks at 141 and 143 ppm are nearly directly comparable. The $\epsilon$-adduct at 141 ppm shows a somewhat longer $T_1$ than the $\delta$-adduct in both cases. This observation fits with the results found previously with the same (unenriched) chemical modification of the protein (1), in which the histidine C$\alpha$ and C$\delta$ resonances showed $T_1$ values of 86 and 78 ms, respectively. In that case the experimental error was rather too large for the C$\delta$ to support a firm comparison with the present results. It is interesting to note that where the dicarboxymethylglycine (NH$_2$ terminal) resonance can be seen it shows a $T_1$ value corresponding to that of the $\epsilon$-adduct on the histidine residues.

**Rotational Correlation Times**—The rotational correlation time, $\tau_R$, is the isotropic motion of the $\alpha$ carbons in the native harbor...
seal myoglobins can be computed from the reported $T_1$ values (1) as either 3.5 or 17 ms according to the solution of a function yielding two real values (7, 8). A similar calculation for the denatured harbor seal apomyoglobin, based on a value (Ref. 1, Table II) of 52 ms for $T_1$, yields a solution for $\tau_{\alpha}$ of approximately 1.0 ns that can be selected with confidence on the basis of line width (1). This value is very close to that previously reported for denatured ribonuclease A (7). The $T_1$ for the $\alpha$ carbon nuclei in the denatured protein is dominated by the correlation time of segmental motion of the backbone (28).

The same treatment can be extended as a crude approximation to yield $\tau_{e\alpha}$ values (7, 8) for the enriched carboxymethyl adducts of 1.5 ns for the native harbor seal protein. The companion $\tau_{e\alpha}$ value of 29 ns is excluded on the basis of careful observation of line width (Fig. 2) and signal intensity (27). In the denatured carboxymethyl protein the adducts on $N^\delta$ and $N^\epsilon$ gave $\tau_{e\alpha}$ values by this treatment of 0.33 and 0.26 ns, respectively. Except in those cases where a number of rotational correlation times are required to interpret $T_1$, the value of $\tau_{e\alpha}$ can be taken as a measure of relative motion (7, 8, 22, 24, 27, 28).

The above simple computations argue that the methylene carbons of the adducts undergo the same correlation time compared to the $\alpha$ carbons when either state of the protein is considered. Making a comparison in terms of $T_1$ values for carbon nuclei in the unmodified myoglobins and the unenriched carboxymethyl derivatives (1), it can be seen that the adducts fall generally between values characteristic of $\beta$ and $\gamma$ carbons. The methylene carbons of the adducts, however, are more equivalent to $\delta$ carbons in terms of their potential degrees of freedom.

The treatment of Doddrell et al. (27) is properly limited to the first degree of freedom of motion relative to a rigid backbone. It is suitable for the appropriate cases of extended rigid side chain groups (24, 28). The present case requires a more elaborate analysis such as that proposed by Levine et al. (32), for which it would be necessary to have more detailed knowledge of the relaxation behavior of all carbon nuclei in the side chain.

By comparison with an alkyl side chain, the bulky aromatic ring of the modified histidine side chain in the protein may be expected to be more hindered in its internal rotational motion. This effect could have the observed consequence of putting the methylene adduct into the $T_1$ class of $\beta$ or $\gamma$ carbon rather than of a $\delta$ carbon, especially in view of the ranges of $\tau_{e\alpha}$ involved (27). Indeed, for the aromatic ring of phenylalanine in these myoglobins there is evidence from the magnitudes of the $T_1$ values that motion about the $C^\delta-C^\gamma$ bond axis is not dominant. When such motion dominates, $T_1$ values for $C^\delta$ and $C^\gamma$ may be expected to be distinctly greater than for $C^\beta$ (33, 34). The basis for that difference would depend on the failure of such motion to modulate the $^{13}$C-$^{15}$N dipole-dipole interaction (33). For phenylalanine residues in the native protein the $T_1$ values (Ref. 1, Table II) for $C^\beta$ and $C^\gamma$ are 40 ms, and for $C^\delta$, 46 ms; the corresponding values for the denatured protein are 101 and 107 ms, respectively. Clearly, other types of motion dominate that about the $C^\delta-C^\gamma$ bond axis in these cases.

On sterically grounds the imidazole-bearing side chain of histidine may be expected to have comparable motional restraints to those of the phenylalanine. In terms of range of $T_1$ values in the denatured case for the imidazole $C^\delta$ and $C^\epsilon$ (Ref. 1, Table II) this prediction is borne out. Within the expected range of contributions to $\tau_{e\alpha}$, if the motion about the $C^\delta-C^\gamma$ bond axis were dominant a clear distinction between $T_1$ values for $C^\beta$ and $C^\delta$ would be expected (35). Such a distinction is not observed. If the correlation time for rotation about the $C^\beta-C^\gamma$ axis is sufficiently long that it does not contribute importantly to the $T_1$ values of the imidazole ring, then a calculation may be appropriate to interpret the $T_1$ values for the imidazole ring carbons in terms of just two correlation times. These correlation times are the $\tau_\alpha$ for the isotropic motion of the $\alpha$ carbons and the $\tau_\gamma$ for the internal motion about the $C^\delta-C^\gamma$ bond axis (27). From this computation $\tau_\gamma$ for the imidazole ring is approximately 0.5 ns for the denatured protein. This argument could be tested if $T_1$ values for the $\beta$ carbons were observable.

Enough degrees of freedom have been shown to be in play in the nanosecond range of correlation time that the motional behavior of the enriched adducts in the denatured protein must be described in terms of contributions involving all the correlation times. Provided that segmental motion of the backbone and internal rotation about the $C^\delta-C^\gamma$ bond axis are dominant, their correlation times should dominate to the same extent the $T_1$ values of the enriched adducts. The significant differences between $T_1$ for the two enriched methylene carbon nuclei listed for the denatured cases in Table III probably depend, therefore, on differences in internal motion about the C-N bonds. On a general steric grounds greater freedom of rotation is to be expected for the $\epsilon$-carboxymethyl adduct.

A corresponding analysis of the native case is hampered by the uncertainty in choice of $\tau_{e\alpha}$ value and the poorer definition in the spectra. For example, detailed information about the ring motion is not available. Considering the elements of restricted motion characteristic of the native structure, the interpretation from the $\tau_{e\alpha}$ values of increased internal motion for the adducts relative to the $\alpha$ carbons is probably justified. It is interesting that the $T_1$ values for the enriched $\delta$- and $\epsilon$-methylene carbon nuclei are indistinguishable in the case. More detailed specific enrichment with $^{13}$C is very desirable.

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