Some Interrelations between Carboxymethylation and Heme Reactions in Sperm Whale Myoglobin*

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SUMMARY

The effect of carboxymethylation of sperm whale ferrimyoglobin on the conversion of the protein to the deoxymyoglobin (ferromyoglobin), oxymyoglobin, and carboxymyoglobin forms was studied. Judged by observations on the absorption spectra and on optical rotation at 233 mp, the conversions to the other heme states were largely unaffected by carboxymethylation to the extent of an average of 7 out of the 12 histidine residues; this left an average of 5 histidine residues unchanged. If the carboxymethylation reaction was carried farther, however, clear evidence of altered properties was obtained. Some evidence was obtained which implied that formation of the carboxymyoglobin derivative could drive the somewhat altered structure back to a form close to the native structure.

The course of carboxymethylation of ferromyoglobin and carboxymyoglobin was also followed. In both cases the reaction followed much the same course as that with ferrimyoglobin. Under comparable conditions slightly more histidine residues proved to be modified in the reaction with carboxymyoglobin than with ferrimyoglobin, an observation reflected in a higher content of 1-carboxymethylhistidine. It is suggested that the difference in content of 1-carboxymethylhistidine may imply a greater reactivity of the E7 histidine residue.

With the exception of the possibility raised above of a slightly higher reactivity at the nitrogen 1 of a histidine residue in the carboxymyoglobin form, the results indicate very similar reactivity patterns of exposed histidine residues in ferrimyoglobin and in ferromyoglobin.

Because x-ray diffraction analysis of protein crystals yields far more accurate and complete structural information than any measurements that can as yet be made on a protein in solution, it is important to refine as much as possible the available methods for comparing structures in the crystalline and dissolved states. Among the methods used to compare myoglobin in the two states are measurements of radius of gyration (2, 3), of relative reactivity of exposed and buried histidyl residues (4-6), and of proximity of lysyl side chains (7). These methods all indicate close similarity between the crystalline and dissolved structures. Furthermore, the native structure in solution is thermodynamically stable, as judged from reconstitution of ferrimyoglobin from heme and apoprotein (8, 9), and the reversibility of denaturation by heat (10), extremes of pH (11, 12), or metal ions (13-16).

To judge from deuterium exchange studies the compact native structure is occasionally lost even under conditions of high overall stability (17, 18). However, the compact structure appears to be sufficiently predominant so that the apparent helix content, for example, is not sensitive to variations in ionic strength, pH, and temperature within certain limits (8, 9, 19). These limits are somewhat narrowed following carboxymethylation (11). It is quite possible that a more open structure is more frequently formed during the carboxymethylation process in which as many as 6, 7, or 8-histidine residues are converted into carboxymethyl derivatives (5, 6). In that case the converted residues might include some that become sufficiently exposed to undergo reaction only as a result of reaction elsewhere in the molecule. This possibility was recognized from the beginning of the work (5). It was supported by the observations that the carboxymethylation reaction could proceed under vigorous conditions to the point of frank denaturation, and that titration studies on the unmodified protein indicated somewhat fewer exposed histidine residues than are apparent from carboxymethylation (4, 5).

The present paper reports, first, the effect of carrying the carboxymethylation process to various stages on the ease and completeness of conversion of ferriMb to forms other than ferriMb, oxyMb, and carboxyMb (19). Noticeable changes appear to be postponed to the later stages of carboxymethylation. Secundly, it reports a comparison of the course of carboxymethylation of ferriMb with that of several derivatives, especially carboxyMb, a derivative that under some conditions is more stable than ferriMb (14). The results are closely similar; only one possibly significant difference is apparent.

* The abbreviations used are: ferriMb, ferrimyoglobin; ferroMb, ferromyoglobin (deoxygenated); oxyMb, oxymyoglobin; and carboxyMb, carbon monoxide myoglobin; and Mb, myoglobin.
EXPERIMENTAL PROCEDURE

The preparations of sperm whale Mb, ultracentrifugation, and routine procedures, such as measurement of pH and amino acid analysis, were identical with or similar to those previously used (4-6, 9, 19). The heme was removed before hydrolysis with methyl ethyl ketone (20, 21). The hydrolysis procedure followed that of Moore and Stein (22). All chemicals were reagent grade.

Determination of Molar Extinction Coefficients

The concentrations of ferriMb solutions were determined by measuring dry weights of standard deionized solutions. Drying was carried out at 107°C to constant weight. Absorption spectra were determined on a Cary model 14 pm recording spectrophotometer. Molar extinction coefficients, ε, were computed for a molecular weight of 17,816.

Procedures for reduction to ferroMb, oxygenation to oxyMb, and conversion to carboxyMb followed those already outlined (19), as described in detail below. All measurements on the Fe(II) state were performed after enzymic reduction utilizing DPNH, methylene blue, and methemoglobin reductase (diaphorase, Calbiochem). The reduction procedure described by Benesch, Benesch, and MacDuff for hemoglobin (23) was again followed (19). The two previously described alternative ways of reaching the Fe(II) state, by dithionite reduction and direct isolation (19), were not employed. Dithionite reduction was inferior to enzymic reduction in the stability of the oxyMb formed subsequently. However, the restriction to enzymic reduction had these disadvantages: the reductase system absorbed too strongly in the ultraviolet region for measurements of ε at 280 nm, and, more seriously, precluded measurements of mean residue rotation at 233 nm. Concentrations of the ferriMb form were referred to the value of 9000 for ε at 503 nm, applicable in the pH range 6 to 7.3 (19). A spectrophotometric cell was attached to a modified Warburg flask with side arms which in turn was connected to a Warburg manometer (Fig. 1). FerriMb solution, 3 ml, in 0.35 to 0.5 M phosphate buffer, pH 7.3, was placed in the main compartment of the flask, while 0.1 ml of methylene blue (0.1 mM), 0.2 ml of DPNH (25 mg per ml), and 0.1 ml of diaphorase (1 mg per ml) in the same buffer, were placed in a side arm. Argon (Matheson Company, gold label purity) bubbled through an alkaline pyrogallol solution was passed through the flask for 2 to 3 min, while the solution was rocked gently. The spectrum of the ferriMb was taken at this point to determine the protein concentration. The contents of the side arm were then tipped into the main compartment, and the passage of argon was continued for another 20 min, after which the absorption spectrum of the ferroMb was recorded. This equilibration period was found to be sufficient. The oxyMb spectrum was obtained by flushing with oxygen for 1 min. Special circumstances are described in the tables in which results are presented.

Carboxymethylation of Protein Crystals

Three preparations of carboxymethyl Mb allowed to react in the crystalline state were studied.

Preparation G—This was prepared by crystallizing 2.38 pmoles of ferriMb from (NH₄)₂SO₄ adjusted to pH 6.09 and a final percentage of saturation of 74%. Under these conditions all the protein was converted to the crystalline state. The mother liquor was carefully removed by suction. A solution 0.1 M with respect to sodium bromoacetate and 0.2 M with

containing 0.01 M EDTA. Potassium bromoacetate was added to the reaction mixture, or bromoacetic acid was added to the phosphate buffer with NaOH used to readjust the pH. The concentration of bromoacetate in the final reaction mixture was usually 0.2 M, although 0.3 M was used in some runs. The protein concentration varied between 0.3 and 0.6 mM. One or two extra charges of bromoacetate were added in some cases, with care taken to avoid disturbing the pH. Special circumstances are described in the tables in which results are presented.

Carboxymethylation Procedures in Solution

Previous techniques were applied (5, 6). All reactions were carried out at 23°C in phosphate buffers at pH 6.9 to 7.0. Reaction mixtures were usually made up with 0.5 M phosphate buffer.
respect to phosphate buffer was prepared in saturated ammonium sulfate so that the final pH was 6.95. The carboxymethylation reaction was initiated by treating the crystals with 1.5 ml of the reaction mixture. The solution was drawn off and fresh reaction mixture added after 2, 5, 6, 8, 10, and 18 days. After 20 days of the reaction at 21°, the last batch of reaction mixture was drawn off. The crystals were washed with 2-ml batches of 75% saturated ammonium sulfate, pH 5.9, with renewal of the wash solution after 6, 12, and 24 hours. Part of this batch of carboxymethylated crystals was taken for x-ray diffraction analysis to be reported elsewhere. This analysis proved that a crystalline structure remained. For the present experiments the mother liquor was drawn off, and the crystals were dissolved in a minimum amount of distilled water, placed in No. 18 dialysis tubing, and dialyzed first against phosphate buffer of pH 7 and then against distilled water.

**Preparation N**—Approximately 2 g of ferriMb crystals were grown from ammonium sulfate solution in the pH range of 7.0 to 7.2. The crystals were placed in a small glass tube with a sintered glass support at the bottom. Reaction buffer was added and changed every 1 to 2 days by gentle suction through the sintered glass. Initially the reaction buffer was 3.8 (NH₄)₂SO₄, 0.2 M in bromoacetate, and 0.1 M in the phosphate buffer adjusted to pH 7.0 with KOH. After 5 days, the phosphate concentration was raised to 0.2 M in order to minimize pH fluctuations. Reaction buffer drained from the column was much the same way as the untreated ferriMb. The results for a sample with the values for completely random coil and completely α-helical forms of poly-α-L-glutamic acid, which were taken as -1,700 and -14,200°, respectively (9). The fractional content of helix, \( \alpha_f \), was computed as

\[
\alpha_f = \frac{[\eta]_b + 1,700}{-12,500}
\]

### RESULTS AND DISCUSSION

**Heme Contents of Alkylated Myoglobins**—Under the usual conditions for carboxymethylation, in which 0.2 M bromoacetate was allowed to react at pH 0.9 to 7.0 at 23° for 10 to 12 days, the product obtained was found to resemble the unreduced ferriMb. The results for a variety of carboxymethyl Mb preparations are shown in Table I. The first 3 columns show the remaining histidine content (as average residues per molecule), the time of reaction with bromoacetate, and the special conditions for treatment with bromoacetate. Column 4 lists values of \([\eta]_b\), the mean residue rotation at 233 μm, and Column 5 the fractional helix content computed therefrom (9).

**Preparation P**—The crystals were treated in a column supported by a sintered-glass disc with a reaction mixture composed of 0.2 M sodium bromoacetate and 0.1 M sodium phosphate in saturated (NH₄)₂SO₄, with final adjustment to pH 7 with NaOH. The reaction mixture was pumped through the crystal bed at approximately 1 to 2 ml per hour. Fresh reaction mixture was prepared at intervals of about 4 days. After 29 days at 23°, the reaction mixture was replaced with 3.8 M (NH₄)₂SO₄ solution. After 4 days of washing, the Mb that had been allowed to react was dissolved in water and dialyzed against water. A portion of this Mb was separated and examined microscopically without being dissolved. It was found to have become amorphous.

### Optical Rotatory Dispersion Measurements

Measurements were made on the Bendix polarmatic instrument (460 B002) by the general technique described previously (9). Generally, cells of 1- or 10-mm path length and temperatures of 23° were used. Results are reported as effective mean residue rotation, \([\eta]_b\), and helix contents were calculated directly from \([\eta]_b\) values. The \([\eta]_b\) values for the samples studied were compared with the values for completely random coil and completely α-helical forms of poly-α-L-glutamic acid, which were taken as -1,700 and -14,200°, respectively (9). The fractional content of helix, \( \alpha_f \), was computed as

\[
\alpha_f = \frac{[\eta]_b + 1,700}{-12,500}
\]
except the denatured one. This is especially true for the \( \epsilon \) values at 409 m\( \mu \) in the ferriMb form and at 555 m\( \mu \) in the ferroMb form. The reductase technique yields most reliable values for ferroMb, as well as for the carboxyMb discussed later. The values for 581 m\( \mu \) and 543 m\( \mu \) in the oxyMb form are less reliable, because they are determined by the balance between competing reactions. Even here, the similarities are reasonably close.

The normal or nearly normal values for extinction maxima reflect similar stoichiometry of the conversions of the heme to ferro- and oxy-forms. The normal conversion stoichiometry is found along with normal values for the Soret peak at 409 m\( \mu \) in the ferriMb form, and for the mean residue rotation at 233 m\( \mu \), \([m']_{233}\). Furthermore, the rates of the reductase action under comparable conditions were observed to be similar. These observations are in accord with the view that the carboxymethyl preparations obtained under these conditions are native with respect to the reactivity of the heme. The excellent quality of the preparations allowed to react in the crystalline state (Preparations G and N) is particularly noteworthy.

The results in Table I for the denatured preparation (Preparation I) are quite different. The analysis of this preparation showed 11.1 residues of dicarboxymethylhistidine per molecule of protein, hardly a trace of unreacted histidine, and too little of the monoesterboxymethyl derivatives for computation of their analysis. The content of lysine residues was 17.9 out of a theoretical 19, and of methionine, 1.8 out of 2 (25). Taken as a whole, the analysis fell within the usual limits for the other carboxymethyl Mb preparations except with respect to histidine and its derivatives. The preparation was also similar to Preparations G and N, in that the absence of unreacted ferriMb was shown by paper electrophoresis (5, 24). Like a previously described preparation obtained by carboxymethylation in urea (5, 9), this preparation showed a much lower apparent helix content judged by \([m']_{233}\) (Table I). The usual heme spectrum was greatly modified, with a broader but lower maximum in the Soret region. For example, at 390 m\( \mu \) and 409 m\( \mu \) the extinction coefficients were 5.4 \times 10^4 and 3.6 \times 10^4, respectively, as compared to values of 7.7 \times 10^4 and 16.3 \times 10^4, respectively, for native ferriMb. This preparation did not act as a substrate for the reductase system. As was expected, the reductase could not produce a stable ferroheme form once the protein moiety of myoglobin was altered to the point of loss of the heme-protein relationship.

The values for the control preparation in Table I fall within the range previously reported (9). It will be noted that the results are not identical with those for the separate control applied to the series of experiments in Table II, which, however, also fall within the previous ranges reported (8, 9, 26). The sources of such variability are under study (1).

Table II compares the spectral properties of the ferroMb, oxyMb, and carboxyMb forms of two preparations of carboxymethyl myoglobin differing to a small but significant extent in degree of alkylation. The conditions of the experiment in Table II were chosen to obtain average histidine residue contents on either side of 5. In each case the standard reaction conditions of 0.2 M bromoacetate, at pH 6.9 and 7.0, at 23° in 0.5 M phosphate buffer were maintained. Sample 1 was allowed to react for a total of 11.8 days, with recharging after 8.7 days. Sample 2 represented part of the same reaction mixture allowed to react for a further 8 days after recharging, for a total reaction time of 16.7 days. Dialysis was against 0.02 M KCl followed by distilled water.

The results in Table II list extinction coefficients at characteristic absorbance maxima for the forms ferriMb, ferroMb, oxyMb, and the azide derivative of ferriMb (9). The values of \([m']_{233}\) for the ferriMb forms are shown also in Table II. The analytical results for histidine residues and derivatives are shown in Table III.

The values for Sample 1 are generally close to those for the control. The extinction coefficients range from 91 to 101% of the control values. The values at the Soret wave lengths, 409, 434, 418, 423, and 421 m\( \mu \), are 96, 98, 97, 101, and 95%, respectively, of the control values. In terms of the \([m']_{233}\) values previously obtained with the same instrument, and of the method of estimating apparent helix content previously used (9), the ferriMb form of Sample 1 contained 96% as much

<table>
<thead>
<tr>
<th>Form of myoglobin</th>
<th>Extinctions coefficients at characteristic of derivatives of absorbance maxima</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \lambda )</td>
</tr>
<tr>
<td>Ferrimyoglobin(^a)</td>
<td>409</td>
</tr>
<tr>
<td></td>
<td>555</td>
</tr>
<tr>
<td>Ferromyoglobin</td>
<td>434</td>
</tr>
<tr>
<td>Oxymyoglobin</td>
<td>581</td>
</tr>
<tr>
<td></td>
<td>543</td>
</tr>
<tr>
<td>Carboxyimyoglobin</td>
<td>578</td>
</tr>
<tr>
<td></td>
<td>540</td>
</tr>
<tr>
<td>Azide ferrimyoglobin</td>
<td>423</td>
</tr>
</tbody>
</table>

\(^a\) At wave length of 233 m\( \mu \) the mean residue rotation \([m']\) for ferrimyoglobin is \(-85°\) for Control, \(-805°\) for Sample 1, and \(-708°\) for Sample 2.

### Partial amino acid analysis of samples in Table II

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Control</th>
<th>Sample 1</th>
<th>Sample 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histidine</td>
<td>11.5</td>
<td>5.12</td>
<td>4.68</td>
</tr>
<tr>
<td>1-Carboxymethylhistidine</td>
<td>0</td>
<td>0.26</td>
<td>0.29</td>
</tr>
<tr>
<td>3-Carboxymethylhistidine</td>
<td>0</td>
<td>1.15</td>
<td>1.03</td>
</tr>
<tr>
<td>1,3-Dicarboxymethylhistidine</td>
<td>0</td>
<td>5.21</td>
<td>5.86</td>
</tr>
<tr>
<td>Total</td>
<td>11.5</td>
<td>11.8</td>
<td>11.9</td>
</tr>
</tbody>
</table>

\(^a\) These results show that the ninhydrin color factor for dicarboxymethylhistidine, which was somewhat arbitrarily chosen (5), is reasonably satisfactory.
helix as the control. All these results show that carboxymethylation according to the procedure applied to Sample 1 had hardly any effect on the interaction of the heme moiety with ligands and caused little disruption of the helical conformation.

The values for Sample 2 at the Soret wave lengths, 409, 434, 418, 423, and 421 μm, are, by contrast, 81, 82, 83, 92, and 79% of the control values. The apparent helix content of the ferri form, based on the procedure applied above to Sample 1 (9), was 77% of that of the control. Most of these results indicate a considerable degree of alteration from the native state. The implied fractional alteration actually is greater than the approximately 20% change from the native state, since the altered forms undoubtedly contributed to the absorbance and optical rotatory values. In the case of the optical rotatory values, an approximate limit for the altered form may be taken from the [m’]233 value in Table I for the denatured preparation. The computed apparent helix content for [m’]233 of -4300 is 0.21, which is 85% of the value of 0.56 taken as normal for the unmodified protein (9). On this basis the average alteration in Sample 2 would amount to approximately one-third, while that in Sample 1 might be one-tenth.

The difference between Samples 1 and 2 is least obvious in the case of the carboxy derivative. The extinction coefficient values at 578, 540, and 423 μm were 93, 96, and 92% of the control, respectively, for Sample 2; and 99, 100, and 101% for Sample 1. Compared with the extinction values for the other derivatives, the carboxy derivatives behave much more like the unmodified protein. It is quite possible that formation of the carboxy derivative specifically stabilizes the native or nearly native conformation of the protein. The carboxyMb derivative of the unmodified protein is known to be more stable under some circumstances than the ferriMb (14).

The ability of the carboxy derivative to form in apparently greater yield than the ferroMb from which it was derived is explained by the fact that the carbon monoxide was introduced in the presence of the active reductase system and thus could couple with the reductase system to drive the reduction closer to the completion. Without the stabilization provided by the carbon monoxide, the altered material in the preparation presumably resisted reduction, as did the denatured preparation in Table I. Unfortunately, the presence of the reductase system, which absorbs strongly in the ultraviolet region, precluded measurement of [m’]233 in the carboxyMb derivatives formed in this way. It has been established that the apparent helix content of unmodified carboxyMb, like that of other heme derivatives (9, 26), is identical with that of the ferriMb form (26).

The results of the foregoing experiments on heme derivatives of carboxymethyl Mb preparations may be summarized as follows. The studies reported in Tables I, II, and III indicate that several preparations containing on the average 5 to 7 unaltered histidine residues per molecule can be converted to ferroMb, and oxyMb forms in much the same way as unmodified ferriMb. The conversion was impossible in the case of a preparation in which essentially all of the histidine residues had been modified. A preparation that had been allowed to react with an average content of unaltered histidine residues of slightly less than five per molecule showed clearly lower extinction coefficients and apparent helix content, except when converted to the carboxyMb derivative. The fact that much of the loss of native properties was reversed by formation of the carboxy derivative indicates that, although the more highly carboxymethylated preparation spontaneously assumed a conformation somewhat different from that of the native, it did retain in considerable degree the ability to revert when stabilized by carbon monoxide. A somewhat similar effect of change in apparent helix content, with change in state of the heme iron, has been reported for cytochrome c by Urry and Doty (27, 28).

A more detailed interpretation of the changes induced by long reaction with bromoacetate would center around the heterogeneity of the product. The presence of several electrophoretically distinguishable components in the unmodified ferriMb and its heme derivatives (19, 29, 30) hinders an evaluation of the distribution of products resulting from the carboxymethylation reaction. In the case of Sample 2 in Tables II and III for example, it would be desirable to know whether any significant proportion of the protein had been converted to a content of histidine residues of three or less per molecule. Procedures are being developed for the preparation on an adequate scale of at least the major Mb component as a homogeneous starting material for future work of this kind (1, 24). Without such a preparation the definitive identification of reacted histidine residues, by degradation and separation of peptides (5, 6, 25), is probably unwarranted for the present purpose.

It is tempting to look upon the changes shown by Sample 2 in Tables II and III as representing a stage in the disruption of the native structure that is carried to completion in the denatured preparation (Preparation 1) in Table I. It is interesting that the gradual opening up of the structure that is implied in this case occurred with the protein continuously in an undissolved state. A second example of the denaturation of the protein by treatment of the myoglobin crystals has been obtained, but no such extensive reaction has yet been observed in solution, except in the previously studied case in which urea was introduced. It is possible that the high concentration of ammonium sulfate that was present during the treatment of the crystals was responsible for making the protein less stable (24).

### Table IV

Carboxymethylation of ferromyoglobin and ferrimyoglobin

<table>
<thead>
<tr>
<th>Reaction time for</th>
<th>Partial composition of reacted protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>days</td>
<td>Histidine</td>
</tr>
<tr>
<td>Ferromyoglobin</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>8.2</td>
</tr>
<tr>
<td>Ferrimyoglobin</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>8.2</td>
</tr>
</tbody>
</table>

The reaction conditions were 0.2 M bromoacetate at 23° in 0.5 M phosphate buffer at pH 6.9 to 7.0.

The values of the heme derivatives (19, 29, 30) hinders an evaluation of the distribution of products resulting from the carboxymethylation reaction.
days, and 17.7 at 8 days. The balance was recovered as \( \epsilon \)-monocarboxymethyllysine \({\text{(31)}}\). The conversion of an average of about 1 lysine residue per molecule under these conditions has been observed frequently.

The results for the histidine residues show great similarities for the two forms of Mb. These results support the conclusion that the ferroMb form has essentially the same structure in solution as ferriMb, at least so far as reactivity of histidine residues is concerned. The close similarity of these two forms in the crystalline state has been established by x-ray crystallography \({\text{(32)}}\).

Table V shows a more extensive comparison of the course of carboxymethylation between carboxyMb and ferriMb. The results for ferriMb are similar to those in Table IV. The observed totals of histidine residues ranged between 11.47 and 12.37, with an average of 11.72, and have been normalized to 12. The results show that, in the early stages with both forms of the protein, the content of 3-carboxymethylhistidine goes through a small maximum. In the later time periods covered, the accumulation of dicarboxymethylhistidine is definitely at the expense of the monocarboxymethyl forms. In other words, after 4 or 5 days the frequency of second alkylation reactions at already modified residues exceeds that of initial attack on unreacted residues. The over-all similarities between the two forms of myoglobin again suggest that the structures differ little in solution, at least with respect to reactivity of histidine residues. The comparisons in Table V are probably more reliable than those in Table IV because the carboxy derivative is more resistant than the ferro derivative to minute contamination with \( \alpha \)-amino during the carboxymethylation reaction.

A point of difference between the two heme derivatives in Table V is more clearly seen in Fig. 2. Much of the consistently lower histidine content of the carboxy derivative is reflected in a higher content of 1-carboxymethylhistidine. It is tempting to ascribe this difference to a difference in reactivity at the nitrogen 1 of the E7 histidine residue. In ferriMb this residue is linked by a hydrogen bond from the nitrogen 3 to a water molecule bound to the heme iron atom. Stryer, Kendrew, and Watson \({\text{(33)}}\) have made the point that in the ferriMb structure the linkage at the nitrogen 3 leaves the nitrogen 1 protonated as a consequence. They further suggest that the proton-bearing role of the nitrogen atoms 1 and 3 are reversed when azide replaces the iron-bound water. Whether or not the carbon monoxide derivative forms a hydrogen bond with the nitrogen 3, it is reasonable to expect that the restriction of the 1-nitrogen to the protonated state would be relieved. A direct study of the azide derivatives of ferriMb is not possible because of the reactivity of azide toward bromoacetate. The foregoing hypothesis, that a relatively high reactivity at the nitrogen 1 of residue E7 might account for the difference between the results for the carboxyMb and ferriMb forms, should be tested by direct isolation of appropriate peptides from the two types of preparation \({\text{(25, 34)}}\).

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Dr. J. F. Bonner kindly provided some reagents and advice.

**Table V**

**Carboxymethylation of carboxymyoglobin and ferrimygoglobin**

The reaction conditions were 0.2 m bromoacetate at 23° in 0.5 m phosphate buffer at pH 6.9 to 7.0.

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<tr>
<td>days</td>
<td>Histidine</td>
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<td>1.7</td>
<td>7.76</td>
</tr>
<tr>
<td>3.8</td>
<td>6.22</td>
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<tr>
<td>5.7</td>
<td>6.66</td>
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<td>7.7</td>
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<td>3.8</td>
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