Carboxymethylation of Sperm Whale Metmyoglobin

REACTIVITY OF THE ADJACENT HISTIDINE RESIDUES*

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The reactivities of the imidazole groups of histidyl residues in sperm whale myoglobin are being studied systematically to achieve a correlation with three-dimensional structure (1, 2). In the sequence between the E- and F-helices there occur 2 histidyl residues, EF4 and EF5, that can be isolated in the same tryptic peptide (3, 4).† It has been shown previously that treatment with bromoacetate before tryptic digestion yields a corresponding peptide differing only in that, on the average, 1 histidyl residue is changed to the dicarboxymethyl derivative (1). The present report establishes that the residue EF4 is reactive towards bromoacetate and EF5 is not.

EXPERIMENTAL PROCEDURE

With the following exceptions, the materials and techniques employed have been described (1).

Digestions with Leucine Aminopeptidase—Leucine aminopeptidase, obtained from Worthington Biochemical Corporation, was used to degrade the carboxymethyl peptide under study, and the order of release of the first few amino acids determined. The enzyme was dissolved in 0.5 M Tris-hydrochloride buffer, pH 8.5. Between 0.05 and 0.10 mg of leucine aminopeptidase was added to 0.2 to 0.5 pmole of the peptide. The final incubation mixture contained 0.1 M Tris and 0.001 M MgCl₂ at 38°. In 1 hour this reaction mixture brought about almost complete release of amino acids from the peptide described previously (1) that corresponds to the sequence F5 to FG2, Ala-GluNH₂-Ser-His-Ala-Thr-Lys (4). No free amino acids could be detected in the enzyme preparation without added substrate.

Chromatography and Electrophoresis—The amino acids released by leucine aminopeptidase digestion were identified by descending chromatography on Whatman No. 1 paper that was equilibrated for 2 hours in an atmosphere of the aqueous phase of 1-butanol-acetic acid-water (4:1:5 by volume) before development with the organic phase. The color development with ninhydrin (by weight) and 1.5% acetic acid and 0.75% pyridine was done by dipping the paper in a solution containing 0.5% ninhydrin-positive material.

RESULTS AND DISCUSSION

Carboxymethylation of Metmyoglobin—Sperm whale metMb³ was allowed to react with 0.2 M bromoacetate at pH 7.0 in 1 M phosphate buffer at 23°. After the mixture had been allowed to react for 4 days, a fresh charge of bromoacetate was added. After a further reaction period of 7 days the preparation was dialyzed against water for 4 days, and again treated with two charges of 0.2 M bromoacetate for periods of 7 days each. After dialysis to remove salts, the preparation was analyzed and found to contain 3.7 unchanged histidyl residues per molecule, approximately 0.6 residue of 3-carboxymethyl derivative, and 7.4 residues of dicarboxymethyl derivative (1).⁴

The absorption spectrum showed quantitative differences from less highly reacted metMb, and some of the material was much more easily precipitated from ammonium sulfate solution. Preliminary measurements of optical rotatory dispersion showed qualitatively the same behavior as either the unmodified protein or less highly reacted material, but its quantitative behavior differed considerably. A detailed correlation of changes in spectrum, solubility, titration behavior, and optical rotatory dispersion with extreme degrees of carboxymethylation is being carried out. The present highly reacted preparation was chosen as a source of the modified peptide in order to minimize the

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³ Dr. W. H. Stein kindly supplied the procedure in advance of publication.

⁴ The abbreviation used is: metMb, metmyoglobin.

This preparation has been alluded to briefly before in Footnote 3 of Banaszak et al. (1).
possibility that any significant amounts of freely reactive histidyl residues might have remained unreacted.

Isolation of Carboxymethyl Peptide—The tryptic peptide corresponding to the sequence EF2 to F4 was isolated and purified by chromatography as described previously for a carboxymethyl-metMb preparation containing approximately 6 residues of unchanged histidine (1). The chromatographic behavior and apparent yield were unchanged from the previous preparation. The level of contamination after chromatography was about 2%. The relative molar composition, with theoretical ratios for the peptide as isolated from untreated metMb given in brackets (4), was: lysine [2], 2.18; histidine [2], 1.03; and dicarboxymethylhistidine [0], 0.81; glutamic acid [2], 2.02; proline [1], 1.17; glycine [1], 0.92; alanine [1], 0.90; and leucine [2], 1.96. The peptide mapping procedure showed a single spot identical with that observed previously (see Reference 1, Fig. 5).

The peptide corresponding to the sequence EF2 to F4 is obtained by an aberrant cleavage, and represents part of peptide Tryp 9 of Edmundson and Hirs (1, 4). The sequence in question is: Lys-Gly-His-Glu-Ala-Glu-Leu-Lys-Pro-Leu, as reported by Edmundson (8).

Degradation by Leucine Aminopeptidase—Since the histidyl residues EF4 and EF5 occur near the NH$_2$-terminal residue in the peptide, it seemed reasonable to follow the release of amino acids by leucine aminopeptidase. The presence of NH$_2$-terminal lysine furthermore suggested that the partially degraded peptides might be more rapidly cleaved than the intact peptide, which, within limits, could be favorable (9). Preliminary experiments followed chromatographically (see "Experimental Procedure") indicated the release of lysine and glycine, in that order. The process was considerably slower than for the peptide corresponding to the sequence F5 to FG2 described above. With larger charges of leucine aminopeptidase the peptide under study could be degraded completely.

When the leucine aminopeptidase degradation was followed by paper chromatography up to a reaction time of 8 hours, it was observed that a spot corresponding to dicarboxymethylhistidine developed very close to the origin after the spots for lysine and glycine had developed. At the same time a spot developed just beyond the dicarboxymethylhistidine. The latter spot gave a positive Pauly test for histidine and presumably represented the residual peptide shorn of the first 3 amino acid residues. It could be distinguished from the dicarboxymethylhistidine by the Pauly test. Furthermore, the dicarboxymethylhistidine forms a gray-blue color with ninhydrin which is easily recognizable in the present system. Under conditions in which the dicarboxymethylhistidine and degraded peptide spots were clearly developed, extremely little free histidine appeared to be present. It might have escaped notice if the sensitive Pauly test had not been used.

The locations of the various products are shown schematically in Fig. 1A. Included is glutamic acid, which is released almost as quickly as histidine when the reaction proceeds further. The initial peptide, which is of course distinguishable from dicarboxymethylhistidine by its reaction with the Pauly reagent, remains at the origin and is not shown in the diagram.

The release by leucine aminopeptidase of dicarboxymethylhistidine prior to histidine was confirmed by analysis of the incubation mixture by high voltage electrophoresis. The distribution observed is shown schematically in Fig. 1B. Samples withdrawn at 8, 10, and 12 hours showed again the rapid release of lysine and glycine, followed by dicarboxymethylhistidine. The latter component was clearly detectable before the release of demonstrable free histidine, at a point where the release of the terminal lysine and the glycine was largely complete.

All the foregoing results of paper chromatography or electrophoresis were based on comparisons of observations of enzymic hydrolysis at 3 to 10 intervals of time.

Implications for Structure of MetMb in Solution—The histidyl residues EF4 and EF5 are unique in metMb in being adjacent in the sequence of the polypeptide chain. Since both residues are subject to carboxymethylation if the native structure is disrupted by exposure to heat or concentrated urea, or by removal of the heme (1), it follows that the lack of reactivity of residue EF5 towards bromoacetate means that this residue is truly masked in the native structure in solution at pH 7. On the other hand, the evidence from chromatography and peptide mapping of tryptic digests of several less highly reacted carboxymethyl-metMb preparations shows that EF4 undergoes reaction with bromoacetate as readily as most other reactive histidyl residues in metMb (1).

Probability that Residue EF5 is Formally Basic—It was shown previously that all or nearly all of the approximately six imidazole groups that are unreactive towards hydrogen ions and p-nitrophenyl acetate in the native, unmodified protein in solution are present in the formally basic, unprotonated condi-

\[ \text{DICARBOXY-METHYL-HISTIDINE} \]

\[ \text{GLUTAMIC ACID} \]

\[ \text{LYSINE} \]

\[ \text{HISTIDINE} \]

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\[ \text{GLUTAMIC ACID} \]

\[ \text{LYSINE} \]

\[ \text{HISTIDINE} \]

\[ \text{PEPTIDE} \]

\[ \text{GLYCINE} \]

Fig. 1. Diagrams of systems for separation and identification of amino acids released from the peptide corresponding to the sequence EF2 to F4 by leucine aminopeptidase. A, descending chromatography on Whatman No. 1 paper in a system prepared from butanol-acetic acid-water (4:1:5 by volume). The $R_F$ of glutamic acid was 0.20. The initial peptide remained at the origin. The spot marked peptide was a degraded, Pauly-positive stage described in the text. The chromatography was carried out for 18 hours at 25°C. B, electrophoresis on Whatman No. 3MM paper in pyridine-acetic acid-water (10:0.4:90 by volume) at 40 volts per cm for 45 minutes. The peptide spot corresponds to the initial substrate peptide.
tion (2). It is quite reasonable to expect that, on grounds of accessibility, those groups that are masked towards hydrogen ions will be masked likewise towards bromoacetate. Indeed, carboxymethyl-metMb preparations, including that studied here, show qualitatively the most important hysteresis characteristics of the titration behavior of unmodified metMb. It is quite probable, therefore, that an unreactive side chain such as that belonging to residue EF5 is masked in the basic form in solution.

Correlation with Crystal Structure—At present the refinement of the x-ray structure has progressed far enough to show that both nitrogen atoms of the imidazole group of EF5 are bonded to other structures in metMb. The side chain of EF4 is less well resolved, but there is as yet no evidence that it is similarly restricted by internal bonding. The structural evidence in the crystalline state (3) correlates with the reactivities of the two residues in solution. The correlation suggests that the structure in that region of the molecule may be very similar in the dissolved and crystalline states. Furthermore, the carboxymethylation of the exposed residue EF4 does not seem to alter the structure in the region of the residue EF5.

The probability that residue EF5 occurs in solution in the formally basic condition also correlates with its apparent bonding in the crystalline state. The nitrogen atom (N-3) of the imidazole group appears to act as a donor in a hydrogen bond to a carboxyl oxygen of the aspartate residue H17. The other nitrogen atom (N-1) is related to an isolated electron density peak in a hydrogen-bonding position which one may take to be the oxygen atom of a water molecule. In this case the water oxygen would act as the donor in the hydrogen bond, thereby restricting the imidazole group to a formally basic state. One may speculate that an imidazole group undergoing the process of entering into a three-dimensional arrangement in the structure of a protein will carry with it a similarly “trapped” water molecule unless it can make some other accommodation of its nucleophilic nitrogen atoms.

Studies are continuing on the carboxymethylation of myoglobin in the crystalline state. The results may provide an even closer link to the crystallographic structure, and provide much of the information needed to describe the chemistry of the imidazole side chains. Previous studies of side chain reactivity have correlated very well with the tertiary structure determined by crystallographic techniques (1, 2, 10, 11).

SUMMARY

A peptide corresponding to the sequence EF2 to F4 in sperm whale myoglobin was isolated from a tryptic digest of carboxymethylated myoglobin. Degradation of the carboxymethyl peptide by leucine aminopeptidase showed that the residue EF4 was in the dicarboxymethyl form whereas EF5 was unchanged histidine. These adjacent residues occur in the interhelical section of the peptide sequence between the E- and F-helices. The implication that EF4 is exposed in solution to an external reagent and EF5 is not corresponds to the crystallographic results of Kendrew and his colleagues.

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