SUMMARY

Crystals of sperm whale ferrimyoglobin were treated with 0.2 M sodium bromoacetate in concentrated ammonium sulfate solution at pH 6.8 for 10 days. The product obtained after dissolving the crystals retained most of the physical properties of the unmodified protein, except for the development of a small number of electrophoretic components representing an increment of about six to eight net negative charges on the average, judged at pH 9.2. The most fully studied preparation contained 6.4, 2.7, 2.0, and 0.8 residue per molecule of histidine, 1,3-dicarboxymethylhistidine, 3-carboxymethylhistidine, and 1-carboxymethylhistidine, respectively. The amino-terminal valyl residue was largely alkylated. Between 1 and 2 residues of lysine were alkylated. No modification of methionine was detected. After removal of heme the modified protein was subjected to cleavage by trypsin and chymotrypsin and the peptides were isolated by chromatography on ion exchange resins and, as required, by paper chromatography or paper electrophoresis. The state of alkylation of 10 of the 12 histidyl residues was established by quantitative estimation of the yields of appropriate tryptic and chymotryptic peptides. The state of residue 12 was determined qualitatively, and that of residue 113 surmised by difference. The following residues were recovered primarily or exclusively as 1,3-dicarboxymethylhistidine: 12, 81, 114, and 116. Residue 116 was recovered partly as 3-carboxymethylhistidine. Residue 36 was recovered in high yield exclusively as 3-carboxymethylhistidine. Without exception these results appear to conform to the implications of the crystalline structure reported by Kendrew and Watson when account is taken of the geometry of interactions between neighboring myoglobin molecules in the crystal lattice.

This paper and the one that follows (3) deal with the comparison of the reactivity toward bromoacetate of sperm whale myoglobin in the crystalline and dissolved states. The particular histidyl residues that undergo alkylation in the two cases are identified by cleavage of the modified proteins and recognition of the separated peptide fragments (4-7).

The primary objective of the present paper is to describe the alkylation pattern in the crystalline state for comparison with the crystallographic structure and to form the basis for the comparison, completed in the following paper, with the alkylation pattern observed in the dissolved state. The crystals studied are the type A monoclinic crystals described by Kendrew and Parrish (8), obtained and alkylated in concentrated ammonium sulfate solutions.

By titration and other procedures it is known that approximately one-half of the 12 histidyl residues in the dissolved protein are sufficiently exposed to the solvent to enter the equilibrium with hydrogen ions, whereas the other histidyl residues are masked (9). Similarly the crystallographic structure indicates the exposure to solvent of certain histidyl residues and the burial of others (10, 11). Since disruption of the protein structure by urea renders all of the histidyl residues subject to dicarboxymethylation by bromoacetate (4, 12, 13), it follows that observed limitations on the reactivity of a given histidyl residue will reflect its masking in the native structure, whether in the crystalline or dissolved state. Since in both states covered histidyl residues distributed over the protein structure are found in both the exposed and masked classes, considerable basis for structural comparison is developed by this one chemical modification technique.

EXPERIMENTAL PROCEDURE

Materials—Frozen sperm whale (Physeter catodon) muscle tissue was obtained from Hvalur (Reykjavik, Iceland) and British Columbia Packers (Vancouver). Phenylisothiocyanate, trifluoroacetic acid, bromoacetic acid, and 2-amino-2-methyl-1,3-propanediol were purchased from Eastman, and the bromoacetic acid was recrystallized from diethyl ether with chilled petroleum ether. Pyridine was redistilled over solid ninhydrin (1 g per liter) before use. DEAE-Sephadex was obtained from Pharmacia. Carbowax (polyethylene glycol Compound 20 M) was purchased from Union Carbide. Dowex 50-X8 was obtained from Agar Instruments (Spinco Division) as amino acid analyzer resin AA-15. Dowex 1-X2 was purchased from Bio-Rad as AG 1-X2.
Twice recrystallized trypsin ( Worthington, TRL 6JA) was treated prior to use with tosyl-1-phenylalanethylchloromethane (Calbiochem) as described by Kostka and Carpenter (14). Twice recrystallized chymotrypsin (Worthington) was used without further treatment.

Aponoglyoglobin from the alkylated myoglobin derivative was obtained by low temperature (−20°) 2-butanol extraction as described by Breslow (15).

All other chemicals were reagent grade.

Preparation of Main Fraction Sperm Whale Myoglobin—The frozen sperm whale muscle tissue was partially thawed and diced into 0.5-inch cubes. One and one-half volumes of 65% saturated ammonium sulfate were added to the diced meat and the tissue was homogenized to a slurry by intermittent blending at 4° in a Waring Blendor. The homogenized material was centrifuged at 7000 to 8000 rpm for 30 min and the supernantant was collected. This supernatant, which contained primarily oxymyoglobin in freshly frozen meat, was oxidized with 2 eq. of solid K₃Fe(CN)₆.

The ferrimyoglobin was then dialyzed exhaustively at 4° against freshly frozen meat, was oxidized with 2 eq of solid K₃Fe(CN)₆. The final ammonium sulfate concentration of between 68 and 70% saturation by careful addition of saturated ammonium sulfate to the dialyzed solution was accomplished at room temperature on a column (4.5 × 60 cm) of DEAE-Sephadex equilibrated with 0.03 M 2-amino-2-methyl-1,3-propanediol buffer at pH 8.70. Separation of the principal myoglobin component (70 to 80% of the total myoglobin in freshly frozen tissue) was accomplished by gravity flow elution at 4° in a Waring Blendor. The homogenized material was centrifuged at 4° to 200 ml per hour. The principal myoglobin component was the earliest heme-containing fraction eluted from the column.

The anion exchange resin was specifically chosen for myoglobin fractionation because it allowed operation over a slightly alkaline pH range in which myoglobin enjoys its greatest stability. The potential for isolating pure oxymyoglobin is an important feature of this preparative procedure. Very slight changes in the conditions (e.g. elution at 4° with 0.03 M 2-amino-2-methyl-1,3-propanediol buffer, pH 8.8) yield stable oxymyoglobin (16, 17). The amine buffer presumably is helpful in forming complexes with trace metal ions.

Myoglobin Crystallization—The ferrimyoglobin was deionized on a column (1.8 × 15 cm) of Rexyn IRG-501 (Fisher) mixed bed resin. The dilute protein solution was then placed in boiled dialysis tubing and concentrated by surrounding the bags with flakes of Carbowax. Concentration was performed at 4° to several hours or until the myoglobin concentration reached 4 to 6%. The myoglobin concentrate was dialyzed against water and finally equilibrated to room temperature.

Crystals were obtained by bringing the protein solution to a final ammonium sulfate concentration of between 68 and 70% saturation by careful addition of saturated ammonium sulfate solution at pH 6.7 to 6.8. Selection of a final salt concentration depended upon the concentration of the myoglobin and the size of the crystals desired. This study was performed on crystals averaging more than 0.5 mm on a side. The type A monoclinic crystals (8) were generally formed within 1 to 3 weeks in capped vials at room temperature. If the initial concentration of ammonium sulfate was too high, precipitation was observed within a few hours.

Crystals were harvested by drawing off the clear supernatant and washing the crystalline protein with saturated ammonium sulfate at pH 6.8. The crystals were then transferred in a minimal volume to the jacketed reaction vessel used for the alkylation reaction.

Reaction of Crystalline Metmyoglobin with Bromoacetate—A solution of 0.1 M ammonium hydroxide was delivered by a Radiometer model TTT1 pH-stat set at pH 6.8 to the unbuffered suspension of myoglobin crystals in ammonium sulfate and sodium bromoacetate. Approximately 600 mg of washed crystals were suspended in 15 ml of 0.02 M bromoacetate made up in saturated ammonium sulfate at pH 6.8 and 25°. The reaction vessel was surrounded with aluminum foil and a scrubbed stream of purified nitrogen was directed over the surface of the liquid. After 2 days, the reaction rate decreased to the point that the pH could be maintained adequately by frequent manual additions of 0.1 N ammonium hydroxide to the reaction mixture. Samples of crystals were taken periodically for amino acid analysis. After 10 days the reaction was terminated by washing the crystals with saturated ammonium sulfate, and the crystalline myoglobin was redissolved in deionized water. Following exhaustive dialysis, aliquots were taken for physical characterization of the modified protein. The remainder was taken for tryptic and chymotryptic digestions and for peptide analysis.

Tryptic and Chymotryptic Digestion of Crystal-alkylated Metmyoglobin—Approximately 600 mg of the modified myoglobin were freed of heme and lyophilized. A 132-mg portion of the globin was dissolved in 15 ml of water and boiled for 15 min to denature the protein. Following the heat treatment the protein was placed in a jacketed vessel and titrated to pH 8.7; then hydrolysis was performed with 1.5% w/w of trypsin for 24 hours at 37°. The hydrolysate was titrated to pH 3.7 and allowed to stand for 12 hours at 4° before the insoluble peptides were removed by centrifugation. The supernatant was titrated to a final pH of 2.2 with 1 N HCl and applied to a Dowex 50-X8 column as described below.

The procedure for chymotryptic digestion of alkylated globin was identical with the tryptic digestion procedure except that the hydrolysis of 264 mg of the protein was performed at pH 8.5 and in the presence of 0.001 M CaCl₂. The chymotryptic hydrolysate was titrated directly to pH 2.2 and the entire mixture was applied to the Dowex 50-X8 preparative column.

Chromatography on Dowex 50-X8 and Dowex 1-X2—The initial separation of tryptic peptides from the modified myoglobin was performed on a column (2.0 × 22 cm) of Dowex 50-X8. The column was equilibrated with 0.05 N pyridine acetate,1 pH 2.4. The column was pumped at a rate of 80 ml per hour for 1 hour with starting buffer and then was developed with a triple linear gradient composed of (a) 250 ml of 0.05 N pyridine acetate (pH 2.4) and 250 ml of 0.5 N pyridine acetate (pH 3.2), (b) a 500 ml of 0.5 N pyridine acetate (pH 3.2) and 500 ml of 1.0 N pyridine acetate (pH 4.25), followed finally with (c) 500 ml of 1.0 N pyridine acetate (pH 4.25) and 500 ml of 2.0 N pyridine acetate (pH 5.0).

The initial separation of chymotryptic peptides from the alkylated myoglobin was performed on a column (2.0 × 22 cm) of Dowex 50-X8. This column was developed at a rate of 80 ml per hour with a double linear gradient composed of 500 ml of 0.05 N pyridine acetate and 500 ml of 0.5 N pyridine acetate, pH 3.2, followed by 500 ml of 0.5 N pyridine acetate and 500 ml of 2 N pyridine acetate, pH 5.0. Finally, a stripping solution of 8 N

1 Concentrations of chromatography buffers are expressed in terms of the total content of the basic component.
pyridine acetate, pH 8.0, was pumped for 2 additional hours (18).

Subfractions of the initial Dowex 50 pools were performed on an analytical column (0.9 x 150 cm) of Dowex 1-X2. This column was developed with a linear gradient of 250 ml each of 3% pyridine, 1 n pyridine acetate (pH 6.0), and 2 n pyridine acetate (pH 5.0). The effluent from all columns was continuously monitored by a Technicon peptide AutoAnalyzer adapted for automatic alkaline hydrolysis and ninhydrin color development (19).

Paper Chromatography of Subfractions—Most of the peptide pools from the ion exchange separations were ultimately purified by paper chromatography. Samples representing 0.5 to 1.0 pmole of peptide were applied to Whatman No. 3MM paper. The peptides were developed by spraying with 1% ninhydrin solution and the indicated bands were eluted with either 30% acetic acid or 30% pyridine solution, depending on the solubility character originally observed for the peptide mixture.

Two standard partition systems were used for paper chromatography. Both contained pyridine, acetic acid, n-butyl alcohol, and water, the first in the proportions by volume of 50:15:25, pH 5.2, and the second in the proportions 15:85:80:70, pH 3.2.

Amino Acid Analysis—Protein and peptide samples were hydrolyzed at 110° in evacuated and sealed tubes in the presence of 6 N HCl for 24 hours. Amino acid analysis of the hydrolysates was performed according to the method described by Spackman, Stein, and Moore (20) with a Beckman-Spinco model 120C amino acid analyzer.

The carboxymethylhistidine derivatives were determined quantitatively by the use of ninhydrin color values determined for the 3- and 1,3-dicarboxymethylhistidine (4, 16). The carboxymethyl derivatives of histidine were synthesized by alkylating N-secetylhistidine and isolating the alkylation products by ion exchange chromatography following acid hydrolysis to remove the acetyl protecting group. Color values of 0.97 and 0.93 were found for 3- and 1,3-dicarboxymethylhistidine, respectively, expressed as ratios to the average color constant obtained for the standard amino acids. An inadequate yield of 1-carboxymethylhistidine was obtained, and its color value was assumed to be equivalent to the value for 1,3-dicarboxymethylhistidine on the basis of steric considerations.

Electrophoresis—Aerylamide gel electrophoresis was carried out at pH 9.2 in a 5% acrylamide gel (Cyanamid Co., American Cyanamid Company) developed at 110 to 125 ma for 1.5 hours. The pH 9.2 Triton X-100 buffer contained 2-amino-2-hydroxyethyl-1,3-propanediol, disodium ethylenediaminetetraacetate, and boric acid, 52.6, 4.4, and 2.6 g, respectively, in 5 liters of water. The vertical gel electrophoresis apparatus supplied by the E-C Apparatus Company was used (21, 22). High voltage paper electrophoresis of peptides was performed in pyridineacetate buffer, pH 3.7 (18).

End Group Analyses—The determination of peptide end groups was performed by a modification of the method of Konigsberg and Hill (23). The amino-terminal valine of myoglobin was quantitatively determined before and after alkylation by the cyanate method of Stark and Smyth (24).

RESULTS

Characterization of Crystal-alkylated Myoglobin—The material used for the recognition of the alkylated residues was obtained after exposure of the crystals to bromoacetate for 10 days. Samples were withdrawn for amino acid analysis at frequent intervals, and the results of following the time course of the reaction are presented in the following paper for comparison with the reaction course in solution (3). The present characterization is confined to the final product, and conforms to the observations reported previously (6). In the present work several crystalline preparations were examined with similarly consistent results.

Before and after exposure to bromoacetate the protein crystals appeared normal under the polarizing microscope, and were similar to alkylated preparations which had been shown to be truly crystalline by x-ray diffraction measurements (6). The redissolved crystal-alkylated ferrimyoglobin showed marked similarity to the untreated protein. A comparison of the absorbance and optical rotatory spectral properties is summarized in Table I.

Amino acid analysis of the preparation agreed with the established composition (12, 25) except with respect to the following residues. The numbers of residues per molecule of histidine, 1,3-dicarboxymethylhistidine, 3-carboxymethylhistidine, and 1-carboxymethylhistidine were 6.4, 2.7, 2.0, and 0.8, respectively. These values are close to those of Preparations C and D reported previously (6). The value for lysine was 17.3 residues per molecule instead of 19 (12), reflecting the usual experience that on average 1 to 2 residues are alkylated under the conditions used (3, 4, 6, 20). Furthermore, the value for valine was approximately 0.7 residue per molecule below the full complement of 8 (12). On the other hand, no loss of methionine was noted, nor could decomposition products of alkylated methionine be detected (4, 13).

Analysis for the terminal valyl amino group by the cyanate method (24) resulted in recovery of 16.8%. The value obtained for the control protein not subjected to carboxymethylation was 87.7%. This result established that the terminal e-amino group underwent modification to a large extent, in keeping with the reduced valine recovery mentioned above. The valyl residue is probably converted to a mixture of mono- and dicarboxymethyl products which would contribute to the observed charge heterogeneity, described below.

Electrophoretic analysis revealed several relatively negatively
Fig. 1, A, elution pattern of the soluble tryptic peptides from crystal-alkylated sperm whale ferrimyoglobin on a column (2.0 × 22 cm) of Dowex 50-X8. The digestion mixture was obtained from 132 mg of the alkylated globin. The column was developed with a three-step gradient at a rate of 80 ml per hour as described in the text. Fractions of 6 ml were collected and the column effluent was continuously monitored by automatic alkaline hydrolysis and ninhydrin analysis (19).

B, elution pattern of chymotryptic peptides well separated from the position of the untreated protein. None of the treated protein retained the charge properties of the untreated material, in keeping with previous experience with the solution-alkylated preparations (4, 26). On the basis of a comparison shown in detail in the following paper (3), it was estimated that the three major bands in the carboxymethylated myoglobin bear 6, 7, and 8 net units of negative charge relative to the untreated protein (16, 25–27). As discussed previously (26), the carboxymethylation of a histidyl residue to any one of the three products will count for a unit increment of negative charge at the pH of the electrophoresis.

Initial Separations of Tryptic and Chymotryptic Hydrolysates—The soluble tryptic peptides were initially separated on a column (2.0 × 22 cm) of Dowex 50-X8 with the previously described three-step linear gradient of pyridine acetate buffers. The resolution obtained and the effluent fractions chosen for pooling are indicated in Fig. 1A. The pattern of elution of the chymotryptic peptides is shown in Fig. 1B. In each case further purification followed programs similar to those used for the seal and porpoise myoglobins (1, 13, 28) based on the initial work of Edmundson and Hirs (12, 29, 30). A complication in the present instance derived from the presence of unmodified histidyl residues as well as of the two forms of monocarboxymethylhistidyl residue, instead of the completely dicarboxymethylated forms used in the sequence analysis (1, 12, 13, 28). In particular, peptides containing unmodified histidyl residues consistently eluted from the Dowex 50-X8 resin as broad peaks. The chymotryptic peptides were generally more easily recovered, and presented few difficulties with respect to solubility. Chromatograms of the subfractionation of pools in Fig. 1, A and B, are not shown, but sufficient detail is included in the following text and tables to indicate the procedures used. Table II summarizes the isolation procedures.

In the following presentation of results, the histidyl residues are dealt with in their order in the amino acid sequence (12). In each case the sequential residue number is identified also in the corresponding terms of the nomenclature introduced for the crystallographic structure (10, 11), shown in parentheses in the section heading. In referring to the separated pools and to the isolated peptides, Roman numerals are used for the former and the peptides are denoted as T-1, etc., for tryptic peptides and C-1, etc., for chymotryptic peptides (1, 13, 28). Compositions of isolated peptides are given in Table III, at the bottom of which are listed the numbers of residues in the peptides, their percentage yield, and sequence position. The content of either leucine, alanine, phenylalanine, or glutamic acid was used as a basis for computing molar ratios of component amino acids of the isolated peptides.

Once purified, all histidine-containing peptides of both tryptic and chymotryptic origin could be unambiguously identified on the basis of the composition alone. None of the 12 histidyl residues in myoglobin occurs in a tryptic or a chymotryptic peptide with an amino acid composition identical with any other (12, 29, 30).

Residue 12 (A10)—The state of alkylation of residue 12 was by guest on March 24, 2020

The abbreviations used are: T-, tryptic peptide; C-, chymotryptic peptide.
Fractionation procedures for isolating histidine-containing peptides

Tryptic, T-1, and chymotryptic, C-1, peptides (Column 1) were initially concentrated in the indicated pools (Column 2) from the Dowex 50-X8 columns described in Fig. 1, A and B, respectively. Secondary fractionation systems applied to these pools are identified as follows. Column 3 describes Dowex 1-X2 pools obtained as described in the text, expressed as effluent volume in milliliters embracing clearly discernible peaks in the monitor tracing. Column 4 specifies the choice of standard solvent system for paper chromatography identified as described in the text according to its pH. Column 5 signifies the use of high voltage paper electrophoresis in the pyridine-acetic acid-water solvent, 1:10:289 by volume. nH 3.75.

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### Table II

| Peptide | Dowex 50-X8 pool no. | Secondary systems | Paper chromatography, pH of solvent | Paper electrophoresis used |
|---------|----------------------|-------------------|-----------------------------------|----------------------------|
| T-1     | IV and V             | ml                |                                    |                            |
| C-1     | X                    | 234-264           |                                    |                            |
| T-2     | V                    | 294-330           |                                    |                            |
| C-2     | XII                  | 240-357           | 5.2                                |                            |
| C-2a    | XV                   | 240-357           | 5.2                                |                            |
| C-3     | XXV (strip)          | 240-357           | 5.2                                |                            |
| C-4     | XXI                  | 240-357           | 5.2                                |                            |
| C-4a    | XXII and XXIII       | 240-357           | 5.2                                |                            |
| T-5     | VII and VIII         | 240-357           | 5.2                                |                            |
| C-6     | XIX                  | 240-357           | 5.2                                |                            |
| C-7     | XXX                  | 240-357           | 5.2                                |                            |
| C-8     | XVIII                | 240-357           | 5.2                                |                            |
| T-9     | II                   | 102-180           |                                    |                            |
| T-9a    | V                    | 186-204           |                                    |                            |

Not established by isolation of a pure peptide, but was indicated by the association of a dicarboxymethylhistidine residue with tryptophan in the insoluble fraction separated from the tryptic digest at pH 3.7 as described under "Experimental Procedure." The peptide in question covers residues 1 to 16. The acid-insoluble peptide fraction was dissolved in glacial acetic acid and a 50-μl sample was applied to a silica gel thin layer chromatography plate. The plate was developed for 6 hours with a solvent system of isoamyl alcohol-acetic acid-water, 2:2:1 by volume. One edge of the plate was sprayed with 2% ninhydrin reagent, and the other with Ehrlich's reagent (31) to test for tryptophan. A positive tryptophan test was observed near the origin, corresponding to a weakly positive band with the ninhydrin spray. A positive tryptophan test was observed near the origin, corresponding to a weakly positive band with the ninhydrin spray. A positive tryptophan test was observed near the origin, corresponding to a weakly positive band with the ninhydrin spray. A positive tryptophan test was observed near the origin, corresponding to a weakly positive band with the ninhydrin spray.

One stage of modified Edman degradation (23) yielded a reduction of 0.7 residue of the expected amino acid, lysine. This peptide is obtained from an overcut at the expected cleavage site at leucyl residue 61. Peptide C-4a was identified in the neighboring pools, XXII and XXIII, following paper chromatography with the standard buffer system at pH 5.2, as the predicted cleavage product representing residues 62 to 64.

Residues 21 (B9)—From both the tryptic and chymotryptic peptides T-1 and C-1, Tables II and III, it is clear that histidyl residue 21 was recovered in the unmodified form. Peptide T-1 represented residues 17 to 31. Peptide C-1 represented residues 15 to 20.

Residues 36 (C1)—Three peptides were isolated to establish that histidyl residue 36 was present in the alkylated protein as the 3-carboxymethyl derivative. Peptide T-2 corresponded to residues 35 to 42. Peptide C-2 was similarly isolated by chromatography on Dowex 1-X2, followed by paper chromatography with the standard buffer, pH 5.2. Peptide C-2a was purified directly from an original pool by the same paper chromatography procedure. These two peptides represented the sequences of residues 34 to 40 and 34 to 43, respectively.

Residue 48 (CD6)—Peptide C-3, containing an unmodified histidyl residue, was recovered from the strip pool collected following the preparative Dowex 50-X8 separation of the chymotryptic peptides. After development of the preparative column with 0.05, 0.5, and 2.0 m pyridine acetate buffers, a stripping solution of 8.0 m pyridine acetate, pH 8.0, was applied for 2 hours. Paper chromatography with standard buffer, pH 5.2, was used to separate peptide C-3, representing the residues 47 to 49.

Residue 56 (Cl)—Peptides C-4 and C-4a were found to contain a histidyl residue in the unmodified form. Peptide C-4 was separated by paper chromatography with the standard buffer of pH 3.2. Despite a quite appreciable contamination with glycine, the peptide was identified as corresponding to the sequence 56 to 64. One stage of modified Edman degradation (23) yielded a reduction of 0.7 residue of the expected amino acid, lysine. This peptide is obtained from an overcut at the expected cleavage site at leucyl residue 61. Peptide C-4a was identified in the neighboring pools, XXII and XXIII, following paper chromatography with the standard buffer system at pH 5.2, as the predicted cleavage product representing residues 62 to 64.

Residues 81 and 88 (DF4 and FE5)—The large tryptic peptide T-5 was isolated from combined pools as specified in Table II and purified by high voltage paper electrophoresis in pyridine acetate buffer, pH 3.7 (13). It was recognized by composition as spanning the region of residues 78 to 98. Several related peptides were identified, resulting from cleavage at other points in this lysine-rich peptide. Peptide C-5 was recovered by paper chromatography with the standard buffer at pH 5.2, and identified as representing residues 77 to 86. Equal proportions of histidine and dicarboxymethylhistidine were found. This peptide is related to the tryptic peptide covering residues 79 to 86 which was shown by Banaszak and Gurd (5) to have dicarboxymethylhistidine in position 81 and unmodified histidine in position 82.

Residues 93 and 97 (F8 and FG5)—By difference, the information given above about Peptides T-5 and C-5 indicates that histidyl residues 93 and 97 were both present in the unmodified form. This was confirmed by isolation of individual chymotryptic fragments. Peptide C-6 was isolated by paper chromatography with the standard buffer system at pH 5.2, and was found to represent residues 90 and 93. Peptide C-7 was purified by paper electrophoresis at pH 3.7, and was found to represent residues 94 to 97.
### Table III

Amino acid composition of tryptic and chymotryptic peptides from crystal-alkylated sperm whale myoglobin

| Amino acids                  | T-1 | C-1 | T-2 | C-2 | C-3a | C-3 | C-4a | C-4 |
|------------------------------|-----|-----|-----|-----|------|-----|------|-----|
| Lysine                       | 4.70 (5) | 2.60 (3) | 0.12 | 1.13 (1) | 0.82 (1) | 1.00 (1) |
| Histidine                    | 2.65 (3) | 0.05 (1) | 0.97 (1) | 1.14 (1) | 0.90 (1) | 0.98 (1) |
| Arginine                     | 1.15 (1) | 0.73 (1) | 0.11 | 1.03 (1) | 0.92 (1) | 2.80 (3) | 2.98 (3) |
| Aspartic acid                | 0.95 (1) | 0.16 | 0.97 (1) | 1.12 (1) | 0.82 (1) | 1.06 (1) |
| Threonine                    | 2.76 (3) | 1.73 (2) | 1.10 (1) | 3.05 (3) | 3.12 (3) | 3.00 (3) |
| Serine                       | 0.95 (1) | 0.24 | 1.14 | 0.90 (1) | 3.00 (3) | 0.29 |
| Glutamic acid                | 2.63 (3) | 1.00 (1) | 1.00 (1) | 0.90 (1) | 0.33 |
| Proline                      | 0.11 | 0.12 | 1.00 (1) | 0.12 | 2.63 (3) | 2.00 (2) |
| Valine                       | 0.78 (1) | 0.83 (1) | 0.98 (1) | 1.00 (1) | 0.97 (1) | 1.00 (1) |
| Methionine                   | 2.00 (2) | 1.11 (1) | 0.12 | 1.03 (1) | 0.92 (1) | 2.80 (3) |
| Phenylalanine                | 0.78 (1) | 0.83 (1) | 0.98 (1) | 1.00 (1) | 0.97 (1) | 1.00 (1) |
| Ileucine                     | 1.01 | 0.12 | 0.90 (1) | 0.90 (1) | 0.33 |
| Tyrosine                     | 1.14 (1) | 0.22 | 0.97 (1) | 1.12 (1) | 0.82 (1) | 1.06 (1) |
| 1-Carboxymethylhistidine     | 2.98 (3) | 2.98 (3) | 3.00 (3) | 0.29 |
| 3-Carboxymethylhistidine     | 3.00 (3) | 3.00 (3) | 3.00 (3) | 0.29 |

**Residue 118 (G14)**—No peptide recognized to contain histidyl residue 113 was isolated from either the tryptic or chymotryptic digestion mixtures. By difference this residue can be taken to be alkylated.

**Residue 119 (G17)**—Peptide C-8 was purified by paper chromatography with the standard solvent system at pH 5.2 and identified by composition as covering residues 116 to 123. This peptide sequence contains two positions for histidyl residues, 116 and 119. The separated material contained 0.9 residue of unmodified histidine and 0.7 and 0.4 of the di- and 3-carboxymethyl derivatives, respectively. Both alkylated histidine forms were removed by a single cycle of the modified Edman end group procedure. This result establishes the site of alkylation as residue 116, and further shows that histidyl residue 119 was present in the unmodified form. It is interesting that the two peptides, with residue 116 in each of the two derivative forms, were isolated together. There is precedent for this in the purification of a peptide from alkylated seal myoglobin (1).

**Residue 119 (G14)**—From the results for Peptide C-8, de-
Table IV

Forms of histidine derivatives identified in crystal-alkylated myoglobin

The recoveries of the histidine-containing peptides from alkylated sheep myoglobin were determined on the basis of small reference peptides (Table III). Ferrimyoglobin crystals were treated at pH 6.8 and room temperature for 10 days with 0.2 M sodium bromoacetate in concentrated ammonium sulfate. The partial amino acid analysis of the alkylated myoglobin was as follows, expressed in residues per molecule: histidine, 6.36; dicarboxymethylhistidine, 2.68; 3-carboxymethylhistidine, 1.06; and 1-carboxymethylhistidine, 0.84.

| Primary sequence position | Isolated form of histidine | Chymotryptic peptide yield | Tryptic peptide yield |
|---------------------------|---------------------------|---------------------------|----------------------|
| 113 G 14                  | Dicarboxymethylhistidine  | %                         | %                    |
| 116 G 17                  | Dicarboxymethylhistidine  | 12.0                      | 8.0                  |
| 119 GH 1                  | Free histidine            | 20.1                      | 20.5                 |
|                           | 1-Carboxymethylhistidine  |                           | 23.2                 |

According to Edmundson (12).

According to Watson (11).

Errors, obtained by difference.

The recoveries generally correspond to that observed during the sequence determination on the related porcine myoglobin with similar techniques (1, 28). The pattern given in Table IV corresponds quite well with the overall composition of the crystal-alkylated protein with respect to histidyl derivatives.

Discussion

Penetration of Crystal by Reagent—The electrophoretic mobility of the alkylated protein was found to be entirely different from that of the untreated protein. No electrophoretic band was observed with the mobility of the untreated protein. These observations establish that the reagent penetrated through the crystal, leaving no detectable fraction of the protein unaltered. Furthermore, the high yield (68.6%) observed for the tryptic peptide containing the 3-carboxymethylhistidyl form of residue 36 in itself expresses a lower limit for the completeness of the penetration of the crystal (Table IV). These results are not surprising, considering the size of certain reagents that have been used successfully to achieve a high degree of occupancy in preparing isomorphous derivatives for purposes of crystallographic analysis of myoglobin (32, 33).

Retention of Native Properties after Alkylation—In keeping with earlier experience (6), the optical rotatory dispersion and absorbance spectral properties of the redissolved crystal-alkylated protein were indistinguishable from those of the untreated material, as summarized in Table I. The alkylated protein showed only a few points of difference from the untreated protein in high resolution nuclear magnetic resonance spectroscopy (34), interpreted in terms of protons associated with the heme group in the cyanoferrimyoglobin derivatives (35).

Correlation of Chemical Results with Crystallographic Structure—The correlation of the observed distribution of alkylated residues (Tables III and IV) with the crystalline structure was made in terms of observations on a molecular model (11) and through computations based on the atomic coordinates (11) and the description of the crystal structure (36). The chemical observations are summarized in Table IV. The isolation of free histidine showed lack of reactivity of the particular histidyl residue, and the isolation of the three derivatives showed reactivity with the corresponding imidazole nitrogen atoms. Residue 113 was taken to be dialkylated by difference, and the assignments of residues S1 and S2 were assumed to be as proved for the reaction in solution (5).

The observed distribution of derivatives could be correlated readily with the crystalline protein structure. Direct observation of the model or reference to the description by Watson (11) was sufficient to interpret the positive reactivity of residues 12, 36, 81, 113, 116, and 119, all of which appear to present at least 1 imidazole nitrogen atom to a reagent diffusing in the surrounding solvent. Likewise, the lack of reactivity of residues 24, 82, and 93 is understandable from their internal positions.

To evaluate the possible role of contacts between myoglobin molecules in the crystalline array in blocking access to any histidyl residues, whether exposed or not in the model of the single molecule, recourse was had to a modification of a computer program kindly provided by Dr. Carroll Johnson. A search was made within 10 A of each imidazole nitrogen atom for all atoms in the coordinate list (11), whether in the parent molecule or a constructed through the kindness of Drs. H. C. Watson and J. C. Kendrew in supplying values for coordinates in advance of publication.

The Thermal-Ellipsoid Plot Program for Crystal Structure Illustrations was supplied by the Clearinghouse for Federal Scientific and Technical Information, Washington, D. C.
The results were printed out as distances of separation and were also displayed as stereochemical pairs of drawings (16). Distances less than 4.5 A were taken as too close to allow productive contact between the bromooacetate molecule and an imidazole nitrogen atom. This estimate was based on approximate dimensions from Pauling's tabulations (37). Presumably effective limitations were observed for the imidazole groups of residues 48, 64, and 97. For example, the 2 imidazole nitrogen atoms 1 and 3 in residue 48 fell within 4.5 A of 5 and 7 other atoms, respectively, comprising parts of residues 52, 53, and 56 in the neighboring molecule related by a screw axis to the parent molecule. Inspection of the stereochemical view appeared to confirm that no channel of approach was available to this residue. Residues 64 and 97, which are less clearly exposed in the model of the single myoglobin molecule, were found to be appreciably hindered by the close approach in the crystalline structure of a different neighboring molecule.

**Specificity of Alkylation according to Imidazole Nitrogen Atom—** The isolation in high yield (Table IV) of the 3-carboxymethylhistidine derivative of residue 36 is an example of highly specific alkylation. Observation of the molecular model as well as the results of the computer search predicts the observed selectivity. Likewise, the isolation of some 1-carboxymethylhistidine derivative of residue 119 without any of the 3- or 1,3-carboxymethyl derivatives is clear evidence of preferential reaction at nitrogen 1. The usual pattern of alkylation favors reaction at nitrogen 3 if the peptide contains well exposed histidyl residues, so that the yield of the 1-derivative is rarely comparable to the yields of the other two. The fact that higher yields of the unmodified form of residue 119 were obtained than of the 1-derivative (Table IV) suggests that this residue was partially hindered with respect to reaction even at nitrogen 1. Such a partial restriction is understandable in terms of the molecular model of the discrete myoglobin molecule, rather than of hindrance from a neighboring molecule in the crystal lattice. On the other hand, any limitation in rate of alkylation of residue 116 implied by the isolation of a considerable amount of the 3-derivative as well as of the 1,3-derivative may be ascribed to the close packing of a neighboring molecule rather than to limited exposure at the surface of the myoglobin molecule itself.

**Alkylation of Groups Other than Imidazole—** The reactivity of the α-amino group is readily interpreted in terms of its exposure on the surface of the molecule and of its pK which is low enough to permit reaction of its conjugate base form. The much smaller relative degree of modification of the highly exposed (10, 11) α-amino groups reflects the restriction due to their high pK range. The lack of reactivity of the methionyl residues is in keeping with their internal disposition in the myoglobin molecule.

**Potentials of Technique—** The main purpose of the present study of the alkylation by bromoacetate of sperm whale myoglobin in the crystalline state was to provide a background for the study in the dissolved state reported in the companion paper (3). The results indicate that the pattern of reactivity of the histidyl residues accords with the structure of the crystalline protein in its crystal lattice as determined by crystallographic methods (10, 11). The technique is a valid probe of reactivity of individual groups in this setting, and can be extended with appropriate caution to the dissolved state of the protein. The present results lend validity to the other studies on sperm whale (4, 5) and human (7) myoglobin in solution.

The categorization of the environments of the individual histidyl residues according to the probability of effective steric hindrance by neighboring structures in the alkylation reaction has been aided in the present case by the rather wide differences between the categories implied by the crystalline protein structure (11). For this reason a more elaborate definition of the spatial requirements for the alkylation reaction process has not been attempted. It is possible that quite misleading results could be obtained if certain channels in a crystal lattice were too constricted to allow access of the alkylating agent to an enclave containing an otherwise completely unrestricted histidyl residue.

Other conditions can be pictured under which the application of such a technique to protein crystals could give unreliable results. Leaving aside the obvious requirements (9) that the added reagent should be compatible with the crystallizing solvent and that the reaction product should remain insoluble, possibly with the help of some cross-linking technique, it is essential that any changes in crystal lattice dimension remain small enough so that the appeal to the unperturbed crystallographic structure remains valid (38). It is possible, moreover, that motion of the molecules in the crystalline state might allow sufficient exposure of a normally internal residue to occur intermittently such that the residue would be found to be reactive and so be classed as external. Departures from time average structures in protein crystals are reviewed elsewhere (39). Another possibility stressed earlier in this series of studies is that of consecutive reactions, in which reaction at 1 residue or more favors increased reaction elsewhere (4, 5). The vulnerability of the region of the molecule near residues 119 and 24 is discussed in the companion paper (3).

Attention should be drawn to the recent study by Bello and Nowoswiat (40) on the alkylation of histidine residues 12 and 119 in bovine pancreatic ribonuclease in which the general pattern of reactivity correlated with previous studies on the dissolved protein.

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