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CHARACTERIZATION OF THE ACTIVE CENTER OF HEMERYTHRIN: THE INVOLVEMENT OF AMINO, IMIDAZOLE AND THIOETHER GROUPS

JOYCE ANN MORRISSEY

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CHARACTERIZATION OF THE ACTIVE CENTER OF HEMERYTHRIN:
THE INVOLVEMENT OF AMINO, IMIDAZOLE AND THIOETHER GROUPS

by

Joyce Ann Morrissey

B.S. Ed., Framingham State College, 1961
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A THESIS

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Since it is impossible to isolate one phase of development from the rest, I must acknowledge and thank all those who have been my teachers and mentors throughout my life. It was they who brought me here. My special thanks must go to Gerald L. Klippenstein for all he has done for me as advisor and friend during my years under his tutorage.

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Abstract

Characterization of the Active Center of Hemerythrin:
Involvement of the Amino, Imidazole and Thioether Groups

by

Joyce Ann Morrissey

Hemerythrin is an oxygen carrying, non-heme iron metalloprotein of known primary structure. Little is known of its higher levels of organization, particularly, the structure of its active center. This paper will deal with the role of the amino, imidazole and thioether side chain groups in the active site of the protein.

Chemical modification and acid-base titration techniques were used to study the involvement of potential iron ligands in the iron site. All of the lysines, the N-terminal glycine, the methionine and three of the histidines (residues 34, 77, and 82) were found not to be active site components. Each of these residues could be modified without destroying the properties of the active site. Modification of all twelve amino groups (11 lysines and the N-terminal glycine) was accomplished using methylacetimidate, succinic anhydride and maleic anhydride. Two histidines were found to be available for acid-base titration, titrating with a characteristic heat of ionization but in an abnormally high pH range (6.4-8.6). The methionine and two histidines (residues 34 and 82) were modified with iodoacetamide after prior treatment of the protein with succinic anhydride. A third histidine (residue 77) was modified by the succinic anhydride. Iodoacetamide modification of the remaining four histidines (residues 25, 54, 73 and 101) was accompanied by destruction of the active center. Thus these residues presumably serve as iron ligands.
Although not involved in the active center, lysine and methionine do appear to be related to the maintainence of the tertiary and quaternary structure of the protein. Four of the lysines are apparently involved in subunit interactions since they are less susceptible to modification and less available for titration in hemerythrin octomer than they are in monomer. The methionine seems to be essential to the maintenance of the protein's tertiary conformation as indicated by the fact that denaturation accompanies its modification.
Hemerythrin is an iron containing metalloprotein which serves a respiratory function in certain marine invertebrates. It is found in sipunculids, priapulids and in some annelids and brachiopods. The most intensely investigated hemerythrin is undoubtedly that of the sipunculid Golfingia gouldii. Several other hemerythrins have been studied as well. These include those of the sipunculids Dendrostomum pyroides (Ferrell and Kitto, 1970, 1971) and Sipunculus nudus (Holleman and Biserte, 1958, Boeri and Ghiretti-Magaldi, 1957, Bates et al., 1968). Dendrostomum pyroides hemerythrin has been found to be quite similar to that of Golfingia gouldii in molecular weight, octomeric structure, oxygen binding properties, tryptic digest fingerprints and amino acid sequence. Although the work on Sipunculus nudus hemerythrin is less extensive, it also seems to be comparable to Golfingia gouldii hemerythrin in molecular weight, quaternary structure, and oxygen binding properties.

The Golfingia protein is obtained from the coelomic fluid of the animals where it occurs in red blood cells. In Dendrostomum zostericolum and Siphonosoma ingens, Manwell (1960a, 1960b, 1963) has observed vascular and muscle hemerythrins in addition to the coelomic hemerythrin. These forms of hemerythrin differ from the coelomic in their oxygen binding affinities and, at least in the case of the vascular hemerythrin, seem to be related in oxygen binding affinity to the oxygen tension of the environment.

Hemerythrin as isolated has a molecular weight of 108,000 (Klotz and Keresztes-Nagy, 1963). It has no prosthetic group. It is composed
of eight subunits which are identical, although a few amino acid inter-
change variants do occur (Klippenstein et al., 1968, Manwell, 1963).
The subunits have a molecular weight of 13,500 and are composed of 113
amino acid residues of known sequence (Figure I) (Klippenstein et al.,
1968).

There are two gram atoms of iron per monomer unit each of which
binds one mole of oxygen (Klotz and Keresztes-Nagy, 1963). Thus each
mole of octameric hemerythrin contains 16 gram atoms of iron and carries
8 moles of oxygen. The oxygen binding sites are independent as evidenced
by a Hill coefficient of 1 (Manwell, 1960a).

An equilibrium exists between the octamer and monomer forms of
the protein with dilution favoring the production of monomer (Keresztes-
Nagy et al., 1965b). Complete conversion of the octamer to monomer may
be accomplished through chemical modification of the protein. Modifica-
tion of the protein's single cysteine residue with a suitable mercaptan
reactant such as p-hydroxymercuribenzoate accomplishes this conversion
(Keresztes-Nagy and Klotz, 1963). Partial succinylation also causes
dissociation to monomer (Keresztes-Nagy and Klotz, 1962).

The two physiologically active forms of hemerythrin are the
colorless iron II deoxy form and the violet-pink iron III oxy form.
Oxidation of oxy or deoxy hemerythrin with K₃Fe(CN)₆ converts hemerythrin
to an iron III methemerythrin with additional bound ligands (Keresztes-
Nagy and Klotz, 1965a. Thus, methemerythrin in the aquo, hydroxy, azide,
chloride, fluoride, thiocyanate, and cyanide forms can be prepared. Each
form has a distinct, characteristic spectrum (Figure 2). Garbett et al.,
(1969, 1971a) suggest on the basis of spectral evidence that the iron
associated structure of the various hemerythrin forms is as follows:
Figure 1: The primary structure of Golfingia gouldii hemerythrin.
Gly-Phe-Pro-Ile-Pro-Asp-Pro-Tyr-Val-Asp-Trp-Pro-Ser-Phe-Arg-Thr-Phe-Tyr-Ser-Ile-Ile-Asp-Asp-

Glu-His-Lys-Thr-Leu-Phe-Asn-Gly-Ile-Phe-His-Leu-Ala-Ile-Asp-Asp-Asn-Ala-Asp-Asn-Leu-Gly-Glu-

Leu-Arg-Arg-Cys-Thr-Gly-Lys-His-Phe-Leu-Asn-Gln-Glu-Val-Leu-Met-Gln-Ala-Ser-Gln-Tyr-Gln-Phe-

Tyr-Asp-Glu-His-Lys-Glu-His-Glu-Gly-Phe-Ile-His-Ala-Leu-Asp-Asn-Trp-Lys-Gly-Asp-Val-Lys-

Trp-Ala-Lys-Ser-Trp-Leu-Val-Asn-His-Ile-Lys-Thr-Ile-Asp-Phe-Lys-Tyr-Lys-Gly-Lys-Ile
Figure 2: Absorption spectra of coordination complexes of methemerythrin.

Extinction coefficients are in terms of protein concentration in grams/liter (Keresztes-Nagy and Klotz, 1965a).
These workers contend that in *Golfingia gouldii* oxyhemerythrin the two iron atoms are in nonequivalent environments. This is not true of deoxyhemerythrin and methemerythrin. Each of these forms of the protein has a single but different iron environment. Thus conformational changes seem to be associated with the conversion of one protein form to another. These hypotheses and the structural theory of Garbett *et al.* are based on Mossbauer spectroscopy, magnetic susceptibility and circular dichroism studies of the various hemerythrin forms and of model compounds. Independent Mossbauer and magnetic susceptibility studies of York and Bearden (1970) support these contentions.

Darnall *et al.* (1968) and Garbett *et al.* (1971b) reported the existence of non-iron anion binding sites in the protein. Their work shows that the protein binds perchlorate and nitrate ions strongly and phosphate, borate and carbonate ions weakly. Sulfate, dodecylsulfate, acetate and trichloroacetate ions are not bound at all. The binding site for these small ions seems to be specific. Analysis of the perchlorate form suggests that this site is near the cysteine since perchlorate ions block reactions of cysteine. Since perchlorate also affects the equilibrium and kinetic behavior of the iron, its binding action also suggests...
that the iron site may lie close to the cysteine. The perchlorate ion binding also affects the reactivity of two lysine residues in the metaquo and methydroxy forms of the protein (Garbett et al., 1971b).

Little is known about the nature of the amino acids involved as iron ligands. On the basis of spectral studies, the iron is believed to be octahedrally coordinated. Allowing for an oxy bridge and the other non-protein ligands, one expects as many as eight amino acids to function as ligands. Evidence to date suggests the tyrosine (Rill and Klotz, 1970; York and Fan, 1970, 1971) and histidine (Fan and York, 1969) may be involved. A number of other amino acids, such as lysine, the N-terminal glycine, cysteine, methionine, aspartic acid and glutamic acid, are also potential ligands. The cysteine has been eliminated as a possible ligand since the monomer produced through modification with p-hydroxymercuribenzoate possesses the spectral properties of the octomer.

Because of their importance to multicellular animal life, Reversible oxygen binding systems are of considerable academic and practical interest. This fact is reflected in the extensive published literature dealing with the hemoglobin and myoglobin systems. The literature on the more primitive oxygen carriers, the hemerythrins, and hemocyanins, although less extensive, still reflects a search for an understanding of the principles of oxygen transport. Hemerythin, because of its relative simplicity, should provide direct insight into these principles. Thus knowledge of its structure and the nature of its active center assumes considerable importance. Only through such knowledge will we begin to understand the vital principles governing metalloprotein and hemeprotein mediated oxygen transport. This paper will deal with the role of certain amino acid residues as potential iron ligands in the active center of hemerythrin. Studies on the N-terminal amino acid, the lysines,
methionine and the histidines have thus been undertaken and will be described here.

The amino groups of lysine and the N-terminal amino acid glycine have the potential to serve as iron ligands in hemerythrin. However, on the basis of the low affinity of iron for an amino nitrogen and what is known of other metalloprotein systems this probability is low. The observation of Klotz and Keresztes-Nagy (1965b) that exhaustively-succinylated hemerythrin displays a native hemerythrin spectrum also suggests that lysine and the N-terminal glycine are not involved in the active center. The actual number of modified amino residues, however, was not determined in these studies. Fan and York (1969) reported modification of eleven amino residues with trinitrobenzenesulfonic acid without any resultant loss of iron or change in the 330 nm extinction coefficient. They declared that this eliminated lysine as a possible ligand. There are, however, twelve amino groups in the protein, eleven lysines and the N-terminal amino acid. Thus, the results eliminate all but one amino group as potential iron ligands.

Hemerythrin contains 6.7 histidine residues. The non-integer value is the result of a variant type of hemerythrin (Hemerythrin B) found in pooled blood samples in which histidine #82 is replaced by an asparagine (Klippenstein, unpublished observations). It is presumed that this histidine can not be involved in the active site of the molecule since the spectral properties of the hemerythrin in which it is absent are the same as the normal hemerythrin with seven histidines. The remaining histidines, however, are excellent ligand candidates. Model compounds show that histidine has a high binding affinity for iron (Vallee and Wacker, 1970). Also, histidine is known to be a ligand in
the heme proteins, cytochrome c, hemoglobin and myoglobin (Margoliash and Schejter, 1966) and in the iron metalloprotein system of transferrin (Ross et al., 1968, Aasa and Aesen, 1968) and the zinc systems of liver alcohol dehydrogenase and alkaline phosphatase (Malstrom and Neilands, 1964).

Fan and York (1969) have studied the histidines of hemerythrin through modification with 5-diazo-1-H tetrazole (DHT) and interpret their results to suggest the involvement of four histidines in the active site.

There is only one methionine in hemerythrin but since iron tends to show a high affinity for sulfur ligands, it must be considered as a possible ligand. Although little data on the involvement of methionine in the active center of other metalloprotein systems is available, it has been shown to be involved in the heme system of cytochrome c (Margoliash and Schejter, 1966).

The basic premise for these studies is that it will be possible to evaluate the role of these different amino acid residues in the protein's active center by evaluating the effect of chemical modifications of the side chains of potential iron ligands. A modification that produces a change in the active site will lead to the conclusion that these residues are involved in iron binding whereas a modification that produces no change will exclude residues as possible active site components.

It is commonly assumed that the active site integrity is reflected in the spectral properties of the protein (Rill and Klotz, 1970, Fan and York, 1969, Keresztes-Nagy and Klotz, 1963). These properties may thus be used to monitor the state of the site. In this work the UV-visible spectrum in the 300-600 m\( \mu \) range and the circular dichroism spectrum in the 300-550 m\( \mu \) range are used for this purpose. Circular dichroism in
the 190-240 m\(\mu\) range is used for conformational analysis.

Three modification techniques which show some specificity for amino groups are maleylation, succinylation and amidination. Maleic anhydride selectively reacts with free NH\(_2\) groups (lysine and the terminal amino acid) at pH 8.5-9.5 forming a maleyl amide bond with a half life greater than twenty weeks at 37°C (Butler et al., 1967). This bond is stable above pH 6 but is hydrolyzed at lower pH's, (Butler et al., 1967). Although the net charge of the protein is changed by maleylation, this may be an advantage since charge repulsion may succeed in opening up the molecule and allow for the reaction of internal lysines. Work on muscle aldolase (Sia and Horecker, 1968) and phosphofructokinase (Uyeda, 1969) has demonstrated the successful application of this modification reagent.

Modification by succinylation is quite similar to that by maleylation. Carried out at pH 7-10, a stable succinyl amide bond is formed (Klotz, 1967). The succinyl group differs from the maleyl group in size and degree of conjugation. Succinic anhydride is less selective than the maleic anhydride in that it also reacts with the hydroxyl group of tyrosine (Klotz, 1967). However, the succinyltyrosine linkage will hydrolyze spontaneously in water (Bezkoroviani et al., 1969). Succinylation also tends to cause subunit dissociation (Klotz and Keresztes-Nagy, 1962). This has been demonstrated to occur with hemerythrin at fairly low levels of succinylation. Succinylation also causes changes in viscosity and sedimentation properties suggesting a molecular expansion or unfolding (Habeeb, 1958).

Amidination is also specific for free amino groups. It is the mildest chemical method of modification in that the acetamidino group is small in size and does not change the net charge of the protein. Because
of these properties, amidination is not expected to produce any gross changes in the conformation of the protein. That this is the case is evidenced by the fact that Wofsy and Singer (1962) were able to modify all the amino groups in rabbit antibodies with minimal changes in their physical and antigenic properties. Reynolds (1968) was able to modify all the amino groups of bovine pancreatic ribonuclease. This procedure inactivated the enzyme but its physical properties remained the same and it could be reactivated by the process of deamidination.

Because the bond formed with lysine by each of these reagents is susceptible to complete or partial cleavage during acid hydrolysis, the reaction can be quantitated by further modifying the protein before acid hydrolysis. This can be accomplished by reacting the modified protein in the presence of 6 M guanidinium chloride with 1-fluoro-2,4-dinitrobenzene which will react with all the unmodified amino groups to form a bond which is stable to acid hydrolysis. The resultant dinitrophenyl amino acids may be easily identified and quantitated. Thus by determining the number of dinitrophenylated amino residues, it is possible to calculate by difference those modified by the initial reagent.

Ascertaining the role of histidine in the active center of the protein by modification is more difficult since there are no specific histidine modifying agents. Because the histidine has a unique dissociation constant and heat of ionization, some information can be obtained through protein titration. This approach is also limited, however, because it may only be used successfully with those histidines that are readily accessible to solvent in the native or slightly unfolded protein. With the hope that the strong points of one technique would reinforce the weak points of the other, both these approaches were used with hemerythrin. Protein titrations were employed to evaluate the availability of the
histidines in the native protein. If, upon titration of these histidines, the spectral properties of the protein are not changed, these groups cannot serve as iron ligands. The modification studies, for the most part, employed iodoacetamide. This procedure allows for the confirmation and extension of the titration results. Thus if it were possible to modify residues which were not titratable and still retain the properties of the active site, additional histidine residues could be excluded as iron ligands. Modification of the histidines to form stable products also allows for their identification in the protein structure.

Protein titrations have proven to be a valuable tool in the evaluation of the availability of different residues in protein systems. Thorough reviews of this approach may be found in Nozaki and Tanford (1967) and Tanford (1962). This approach has been used very successfully in studies of hemoglobin, lysozyme, ribonuclease, and bovine serum albumin (Nozaki and Tanford, 1967, Janssen et al., 1970, Tanford et al., 1955, Tanford, 1962). On the basis of the data from titrations, it is possible to calculate the numbers of titratable groups in various pH ranges, their heats of ionization and their intrinsic pK's. Since hemerythrin is stable throughout an extensive pH range, it should lend itself well to titration studies.

As mentioned previously, chemical modification of histidine generally proves to be a difficult task because of the lack of selective reagents. Fan and York (1969) employed 5-diazo-1-H-tetrazole in their study of the response of hemerythrin to histidine modification. However, because of the color of the modified product, the effect of this reagent on the active center becomes difficult to evaluate since changes in the integrity of the active center are observed using spectral properties. This is also true of the modification of histidine with 1-fluoro-2,4-
dinitrobenzene. The reaction of histidines with α-halo acids and their amides is a more desirable modification technique in this regard in that the modified histidines have no UV or visible absorption. In addition, the carboxymethylhistidyl bonds are stable to acid hydrolysis and the end products of the reaction can be separated on an amino acid analyzer, thus enabling one to study the quantitative aspects of the reaction (Grundlach et al., 1959a).

The α-halo acids and amides react with cysteine, methionine and lysine as well as histidine (Gurd, 1967). However, careful selection of reaction conditions increases the selectivity of the reaction. The lysine reaction may be decreased or eliminated by preliminary modification of the lysines with succinic anhydride or some other lysine specific modifying agent. Preliminary specific modification of the cysteine may also be employed. In hemerythrin, however, the reaction of cysteine with α-halo acids and their amides constitutes no problem since it has already been excluded as an active center group. Methionine and histidine remain. The reaction of methionine with α-halo acids and their amides is independent of pH whereas that of histidine is not (Gurd, 1967). The histidine reaction is favored above pH 7 and greatly restricted below pH 6. Thus although these two reactions must be considered concurrently at high pH's, the methionine reaction can be separated from the histidine reaction by also studying the reaction at low pH [values as low as pH 2 may be used (Gurd, 1967)].

The methionine reaction is not as easily quantitated as is the histidine reaction since degradation of carboxyamidomethylmethionine occurs during acid hydrolysis, producing a number of products including methionine (Grundlach et al., 1959b). Oxidation of the unreacted
methionine with performic acid may be carried out before hydrolysis, however. This converts the methionine to methionine sulfone which is stable to acid hydrolysis and can be separated on an amino acid analyzer (Grundlach et al., 1959b, Moore, 1963). This procedure enables one to calculate by difference the amount of carboxyamidomethylmethionine.

Thus, although carboxymethylation is a complex reaction, it is not without advantages. One can exercise selectivity in the reaction by careful control of the reaction conditions or one may study the effect of the concurrent modification of more than one type of residue. This flexibility is useful in studying the hemerythrin system since it enables one to study both the methionine and histidines at the same time, and yet with minor changes in technique, it enables one to separate the reaction of methionine from that of histidine.
METHODS AND MATERIALS

Hemerythrin Preparation

Animals were obtained from Marine Biological Laboratory, Woods Hole, Mass. For each preparation of hemerythrin the coelomic fluid from about 100 *Golfingia gouldii* was collected and kept at 0°. The hemerythrin was prepared according to the method of Groskopf et al. (1966). The coelomic fluid was stirred with a glass rod to facilitate coagulation. The debris from the collection procedure was removed by filtration through glass wool. The red blood cells were separated from the plasma and buffy coat by repeated centrifugation for 10 minutes at 760 g. The buffy coat was removed with a Pasteur pipet. The red cells were then packed by centrifugation for 1 hour at 1000 g and the supernatant removed. All these initial operations were carried out in 3.5% NaCl. The cells were then lysed in 0.4% NaCl. The soluble hemerythrin was separated from the cell debris by centrifugation at 27,000 xg for 45 minutes. The supernatant was dialyzed against ethanol: 0.4% NaCl, 1:4 (v/v). Five hours were allowed for crystallization. The hemerythrin crystals were separated from the solvent by centrifugation for 10 minutes at 1000 g. The crystalline hemerythrin was washed with the ethanol, salt water solution and stored suspended in this medium at -15°.

Methemerythrin

The methemerythrin derivatives were formed from the oxyhemerythrin crystals which were dissolved in 0.1 M Tris acetate buffer, pH 8.0. Potas-
sodium ferricyanide was added to a concentration of 2.2 moles per mole of protein monomer. The protein was allowed to react at 5° for a minimum of two hours. The oxidizing agent was separated from the protein on a Sephadex G-25 column (2.5 x 26 cm). The extent of conversion to metahemerythrin was evaluated by its spectrum. Various salt derivatives of the methemerythrin were prepared by dialysis of this protein overnight against 0.1 M solutions of the desired salt. The concentration of each derivative was determined from the absorption at 280 μm. Hemerythrin has an extinction coefficient of 2.58 l/g cm at 280 μm (Subramanian et al., 1968). This concentration was used to find the extinction coefficients of the various salt derivatives at their characteristic peaks and shoulders between 300 and 600 μm.

**Hemerythrin Monomer**

Hemerythrin monomer was prepared by reacting the appropriate hemerythrin form with a two fold molar excess of p-hydroxymercuribenzoate. This modification reaction was carried out at 0° in a 0.025 M sodium borate buffer, pH 8.6.

**Apoheremerythrin**

The apoprotein form was prepared by prolonged dialysis of methemerythrin monomer (≈5x10⁻⁴M) against a solution 0.1M in sodium phosphate buffer, pH 8.3, 0.1 M in sodium dodecylsulfate (SDS) and 0.1 M in disodium ethylenediaminetetraacetate (EDTA).

**Modification of the Lysine and N-Terminal Glycine**

The same basic method for modification of the amino residues was used in all cases. In most experiments the protein was in its monomer
form. The metazidehemerythrin was modified with increasing quantities of reagent calculated as molar excess with respect to lysine. Upon completion of the reaction the excess reagent was removed by dialysis and the modified protein was examined in the 300-600 μ range on the Carey 15 spectrophotometer. In addition, the circular dichroism of some samples in the 300-500 μ range was examined using a Cary 60 Spectropolarimeter.

The modified protein was then denatured with 6M guanidinium chloride and reacted with 1-fluoro-2,4-dinitrobenzene to modify any residues that were unmodified by the initial reagent. The resulting protein was dialyzed, lyophilized and subjected to acid hydrolysis for 18 hours. The hydrolyzed samples were then analyzed for ε-DNP-lysine on the short column of a Beckman amino acid analyzer model 120C as described by Wofsy and Singer (1963). Amino acid standard solutions containing ε-DNP-lysine were used to quantitate the results of the analysis. DNP-glycine was determined separately by paper chromatography.

Maleylation

The procedure followed for maleylation was essentially that of Sia and Horecker (1968). Their procedure was modified in that the reaction was carried out in 0.025 M sodium borate buffer, pH 9.0. In instances where the octamer form of the protein was used, the solution was made 10mM in mercaptoethanol. The protein solution (0.4 moles/ml) was placed in a small beaker with a magnetic stirrer. The beaker was placed in an ice bath to maintain the temperature at 0°C. The pH was monitored with a single microelectrode connected to a Fisher Accumet expanded scale pH meter. Maleic anhydride was dissolved in acetone to a concentration of 0.05 g/ml. The appropriate concentration of anhydride (a two to thirty
fold molar excess with respect to lysine) was added to the protein in small amounts with a Gilmont micrometer burette with a 2.5 ml capacity (smallest division 0.001 ml). The pH was maintained between 8.5 and 9.5 by the addition of 2M NaOH. After each addition of reagent the pH was allowed to stabilize before the next addition was made. After the reaction was completed the samples were dialyzed overnight at 5° against either 0.025M sodium borate buffer, pH 9.0 or 0.1 M sodium azide solution. Results showed that the sodium azide dialysis was preferable.

**Lowry determination of protein concentration.** Since the 280 μm absorption of the maleylated protein was not a reliable index of the protein concentration due to the absorption of the maleyllysine, it was necessary to determine protein concentration in a different manner. This was done either by estimating the contribution of the maleyllysine to the 280 μm absorption from its 280 μm extinction coefficient or by determining the protein concentration by the Lowry method as modified by Oyama and Eagle (1956). Hemerythrin was used as a standard.

**Succinylation**

The basic procedure for succinylation was that of Klotz (1967). The physical conditions for the experiment were those used in maleylation. The protein was always converted to monomer before reaction. The reaction was carried out either in 0.025M sodium borate buffer, pH 8.6, or in 0.1M sodium azide solution. Small weighed amounts of succinic anhydride were added to achieve the desired molar excess (two to fifteen fold with respect to lysine). The pH was kept between 8 and 9 by the addition of 2M NaOH. After all the succinic anhydride had been added and the pH had stabilized the samples were allowed to stand at 0° for 30 minutes. They
were then dialyzed overnight against 0.1M sodium azide solution at 5°.

Amidination

The reagent, methyl acetimidate, was prepared in accordance with the procedure described by Ludwig and Hunter (1967). The technique employed for modification reaction was essentially that of Ludwig and Hunter (1967), Reynolds (1968) and Wofsy and Singer (1963). Both hemerythrin monomer and octamer were used and in both cases the protein was in 0.025M sodium borate buffer, pH 8.5. Four weighed portions of methyl acetimidate, in relative molar proportions 1:1:1:2 were neutralized with an equimolar amount of 2M NaOH and then added to the protein solution which was adjusted to pH 9.5, 10.0, 8.8 and 9.5 for the respective additions (Ludwig and Hunter 1967). The total molar excess of reagent was 100-1000 fold with respect to lysine. The reaction was allowed to proceed for 30 minutes after each addition of reagent. The pH was controlled by the addition of small amounts of 2M HCl. After the reaction was complete the white precipitate that had formed was removed by centrifugation at 250 g for 15 minutes at 0°C. The modified hemerythrin was then dialyzed against 0.1M sodium azide solution.

Dinitrophenylation

The general dinitrophenylation technique of Porter (1957) and Fraenkel-Conrat et al. (1955) was employed on protein samples containing 0.2-0.4 μmoles of protein. All operations were carried out in the dark. Before dinitrophenylation the protein was made 6M in guanidinium chloride. An equal volume of 5% Na₂CO₃ solution was then added to the protein and the pH adjusted to 9.0 with 2M NaOH. A volume of 1-fluoro-2,4-dinitro-
benzene (10% w/v in ethanol) equal to the original volume of the protein was then added. The reaction mixture was allowed to react for two hours at room temperature with constant stirring. It was then acidified with HCl to precipitate the protein. The excess reagent was extracted with three 25 ml portions of ether. After the extraction, the protein was dialyzed against water in the cold room overnight. The samples were lyophilized and then hydrolyzed with constant boiling HCl at 110°C for 18 hours. After hydrolysis the HCl was evaporated in a vacuum desiccator. Sodium citrate buffer, pH 2.2 (2.5 ml) was added and the resultant solution was analyzed on the amino acid analyzer. The amino acid standard contained 0.1 μmoles of each amino acid and 0.1 μmoles of ε-DNP-lysine. The ε-DNP-lysine elutes from the short column of the amino acid analyzer immediately after arginine (Wofsy and Singer 1963).

**DNP-Glycine Analysis.** Since DNP-glycine is destroyed by the above 18 hr. hydrolysis, it is necessary to analyze for it independently. This may be accomplished by using shorter hydrolysis periods. Thus for this analysis a sample of the dinitrophenylated protein was hydrolyzed at 110°C for 4 hours. The hydrolysate was then extracted three times with ether and the ether layer washed three times with water. The ether was allowed to evaporate and the residue was dissolved in 0.5 ml of ethanol. The sample was then applied to Whatman #3 MM chromatography paper that had previously been washed with 0.5 M potassium phthalate, pH 6.0 and dried. The samples were then chromatographed using a solvent system composed of tertiary amyl alcohol (peroxide free) saturated with 0.5 M potassium phthalate, pH 6.0 (Porter, 1957). The chromatogram was allowed to develop for 24 hours. DNP-glycine dissolved in ethanol was run as a standard.
Hemerythrin Titrations

The basic procedure of Nozaki and Tanford (1967) and Dyson and Noltman (1969) was followed for all titrations. Potassium hydroxide solutions (CO$_2$-free) were standardized against potassium acid phthalate. Hydrochloric acid solution (NH$_4^+$-free) was standardized against the base. The acid and base solutions were made carbon dioxide and ammonia free by boiling glass distilled water for fifteen minutes and then cooling and storing it in bottles stoppered with a Mallcosorb tube (Mallinckrodt) for the carbon dioxide and a citric acid tube for the ammonia.

The protein was titrated in a water jacketed vessel at 5°, 15°, or 25° under a humidified nitrogen atmosphere. The temperature was maintained by a Tamson or Haake water bath. A single glass micro-electrode (Fisher Scientific) was used with a Fisher Accumet expanded scale pH meter. Prior to titration the protein was dialyzed overnight against carbon dioxide-free water. It was then run through an Amberlite MB-3 mixed bed resin to remove any contaminating ions. The isionic protein was allowed to stand for two hours under a nitrogen atmosphere. The protein concentration was measured by its absorption at 280 μm.

Protein concentrations were approximately $1 \times 10^{-4}$M for the limited range titrations and approximately $6 \times 10^{-4}$M for full range titrations.

The pH meter was standardized against freshly prepared buffers (Fisher Scientific) of pH 7.00, 4.00, and 10.00 made with CO$_2$ free water. The sample (3 or 5 ml) was then transferred to the titration vessel and the isionic point was measured. KCl was added to raise the ionic strength to 0.1 μ and the protein was allowed to stand in the titration vessel for 30 minutes. The standard acid and base were diluted to 0.05 M (base, 0.0454 M; acid, 0.0506 M) and made up to an ionic strength of 0.1 μ
with KCl. Base was added to bring the pH of the protein to the desired value and it was titrated to pH 4.5 with acid. The procedure was reversed when the base was used as a titrant. During the titration small increments of acid were added with a Gilmont micrometer buret. The pH was measured after each addition. Following the protein titration a blank of 0.1 μ KCl was titrated within the same pH range. In some cases the spectrum of the protein between 300 and 600 μm was recorded at the lower limit of the titration (pH 4.5).

Full Range Titration

In titrations over the full range, relatively concentrated protein solutions (-6 x 10⁻⁶M) were used. They were made 0.2 μ with KCl and titrated with standard 0.1 M acid and base solutions made up to a total ionic strength of 0.2 μ with KCl. In the region of denaturation the pH was allowed to stabilize before a reading was taken. This sometimes involved a period of over an hour. Before each of these readings the pH meter was restandardized with freshly prepared buffer.

Apohemerythrin Titrations

Apohemerythrin solutions were dialyzed first against 0.1 M NaCl, and then overnight against CO₂-free water. The protein solution was mixed with ion exchange resin for 10 to 15 minutes and the resin then filtered off under nitrogen. The titration procedure was that used with the native hemerythrin in the limited range.

Titrations in Guanidinium Chloride

Hemerythrin was titrated in denaturing media to find out if
unfolding the protein molecule would make more histidine residues available for titration. Guanidinium chloride was the initial choice of a denaturant. Solutions of 1, 3 and 6 M guanidinium chloride (Mann, ultra pure) were titrated with 0.1 M HCl after being raised to pH 9 with KOH in order to obtain correction factors for the protein titrations. Following this protein solutions of the same guanidinium chloride concentrations were titrated under the same conditions. In both cases pH readings were found to be unstable and the amount of titrant consumed in the basic area of the curve was considerable. To minimize this effect the titrations were carried out rapidly. The spectra of the solutions were recorded before and after titrations to pH 5.0.

Titrations in Urea

Because even ultra pure guanidinium chloride had too many titratable groups in the basic region, urea was used as a denaturant. It was prepared at an 8 molar concentration in CO₂ free glass distilled water. The solution was passed through the amberlite mixed bed ion exchange resin and stored under nitrogen. A solution of the 8M urea, 0.1 μ in KCl was titrated with HCl as a standard. The titration curve indicated that it would be an acceptable medium for the titration since the correction factors would be small. A spectrum of this solution in the 280-600 μm range indicated that it would be satisfactory on this basis as well. The 8 molar urea solutions were then diluted with CO₂ free water and with the isionic protein to attain the desired protein and urea concentration. The solutions were made 0.1 μ in KCl and titrated with 0.1M acid or base. After titration the spectra of the samples were recorded in the 300-600 μm range.
Calculations

The Z values at the isionic point were calculated according to the formula $Z = C_{OH^-} - C_{H^+}/C_P$ (Nozaki and Tanford 1967). $(Z = \text{net charge on the protein}, C = \text{concentration}, P = \text{protein})$. At no time was this Z value significant. The volumes of titrant added from the isionic point to any given pH were corrected in terms of the blank titration and the $Z$ values for each pH calculated from the formula $Z = \frac{(\text{volume acid or base - blank correction}) \times \text{molarity protein} \times \text{volume protein}}{\text{concentration}}$.

For the full range titrations the following formula was used to account for dilutions

$$Z = \frac{V_T \times M_T - \left[ \frac{(V_{KCl}) \times (M_T) \times (V_S + V_T)}{V_S + V_{KCl}} \right]}{C_p}$$

- $V_{KCl}$ = volume to titrate 0.2 M KCl
- $V_T$ = volume to titrate protein
- $M_T$ = molarity titrant
- $V_S$ = volume protein sample
- $C_p$ = concentration protein

In the limited range the dilution was not significant allowing for use of the simpler formula.

Histidine-Methionine Modification Studies

Dinitrophenylation

Completely succinylated metazidehemerythrin and native metazide-hemerythrin in 0.1M sodium azide solution were reacted with 1-fluoro-2,4-dinitrobenzene under the conditions described earlier except that denaturation with guanidinium chloride and the addition of NaHCO$_3$ were omitted. The reaction was carried out at 5° to preserve the native configuration. After the reaction the samples were analyzed as described earlier. The
degree of histidine reaction was determined from the quantity of unmodified histidine and the nature of the modification evidenced by the presence of an im-DNP-histidine peak immediately before lysine (Henkart, 1971).

Carboxymethylation

**Preliminary Modification.** By preliminary blocking of hemerythrin's lysine and cysteine residues, the modification with iodoacetamide was limited primarily to amino acid side chains of histidine and methionine. The cysteine was blocked with p-hydroxymercuribenzoate as described earlier. This procedure also converted the protein to its monomer form. The lysines were blocked by succinylation or maleylation. A succinylation or maleylation level was selected which allowed for maintenance of most of the native metazidehemerythrin spectrum and was at the same time sufficient to keep the protein in solution during the iodoacetamide reaction. The succinylation reaction was carried out as described earlier except that the pH was kept between 7 and 8 as opposed to 8 and 9. Quantities of succinic anhydride 4, 6, and 8 times the protein's lysine content were tried. On the basis of the spectrum of the succinylated protein and its stability on subsequent carboxyamidomethylation, six moles of succinic anhydride per mole of lysine in the protein was selected for this preliminary modification. For maleylation, a level of maleic anhydride equal on a molar basis to the protein's lysine content was used. The maleylation reaction conditions were not changed from those described earlier.

**Modification with Iodoacetamide.** The kinetics of the reaction of the succinylated or maleylated protein with iodoacetamide was studied.
The succinylated or maleylated hemerythrin \((1.5 \times 10^{-4} \text{ M})\) in 0.1M Tris cacodylate buffer, pH 8.0 and 0.1 M in NaN\(_3\) was reacted with 0.4 M iodoacetamide at 30° in the dark. Samples were removed at various times between zero and 96 hours for the succinylated protein and zero and 25 hours for the maleylated protein. Each sample was run through a Sephadex G-25 column \((1.5 \times 11 \text{ cm})\) equilibrated with 0.05 M NaN\(_3\) to remove the excess iodoacetamide. The spectrum between 300 and 600 \(\mu\)m and the \(A_{280}\) of each purified sample was determined. The samples were then dialyzed against deionized distilled water. After dialysis 0.1 \(\mu\)moles of each sample was lyophilized, hydrolyzed and analyzed on the amino acid analyzer. The acid hydrolysis differed from that employed with the dinitrophenylated proteins in that two drops of 0.5 M hydrazine were added to each tube to prevent tyrosine destructions (Sanger and Thompson, 1963). The carboxymethylhistidine derivatives produced during acid hydrolysis were quantitated as described by Crestfield et al., 1963. Three carboxymethylhistidine derivatives were noted: 1, 3-dicarboxy-methylhistidine eluting about 18 minutes before aspartic acid, 1-carboxy-methylhistidine eluting with proline and 3-carboxymethylhistidine eluting after alanine. The serine calibration constant multiplied by .955 was used to quantitate 1, 3-dicarboxymethylhistidine and the glycine calibration constant was used for 3-carboxymethylhistidine. The proline contribution (proline calibration constant value \(\times 4 \times \mu\)moles protein) was subtracted from the combination proline + 1-carboxymethylhistidine peak to find the quantity of 1-carboxymethylhistidine. The threonine calibration constant value was then used for this derivative.

A second 0.1 \(\mu\)mole portion of the iodoacetamide-treated succinylated protein was analyzed for its iron content using the o-phenan-
throline method of Rill and Klotz (1970). The circular dichroism of each sample in the 190-250 μ region and of selected samples (4, 18, and 32 hours) in the visible region were also examined. A final portion of each iodoacetamide-treated succinylated sample was oxidized with performic acid according to the procedure of Grundlach et al. (1959) and Moore (1962). These samples were hydrolyzed and analyzed for methionine sulfone in order to determine the amount of methionine modified. This was necessary since carboxyamidomethylmethionine decomposes on hydrolysis.

A pure sample of a variant of *Golfingia gouldii* coelomic hemerythrin was also examined under the same conditions. This variant is different from the major hemerythrin component at five sites in the amino acid sequence including the substitution of an asparagine for histidine at residue 82. Samples were removed over a period of zero to 36 hours of reaction. The spectra in the 300-600 μ range and the A₂₈₀ were recorded. Amino acid analysis of the acid hydrolyzed and the oxidized acid hydrolyzed samples was employed to identify the reacted histidines and methionines.

In order to separate the effects of the methionine reaction from those of the histidine reaction, the carboxyamidomethylation reaction of the succinylated protein was also studied at pH 5.5 using an acetic acid-sodium acetate buffer system. The other conditions of the reaction remained the same. Samples were removed over a period of zero to 26 hours. Precipitation of the protein was observed to begin at 6 hours. The precipitate was removed by centrifugation and the soluble protein examined. As before the 300-600 μ spectrum and A₂₈₀ were recorded and the modified histidines and methionines determined by amino acid analysis of the acid hydrolyzed and the oxidized, acid hydrolyzed protein. The
precipitate from the 26 hour sample was collected, washed, and then subjected to iron analysis for modified residues as above. The concentration of protein in the precipitate used in the iron analysis was determined by dissolving the precipitate in 3.0 ml of 0.1 M NaOH and reading the absorption at 280 μm. From this absorption and a standard curve of 280 μm absorption versus hemerythin concentration in 0.1 M NaOH, the concentration of the protein was determined.

Iron Analysis. The method of Rill and Klotz (1970) was employed. One ml of the protein solution containing approximately 0.1 μmoles of hemerythin was placed in a graduated centrifuge tube. In most cases the exact concentration of the protein was calculated on the basis of the amino acid analysis of the modified hemerythin. To this solution was added 2.5 ml of o-phenanthroline solution (0.025% in 0.5% H₂SO₄), 0.5 ml 10% H₂SO₄ and 0.5 ml 10% hydroxylamine hydrochloride. The pH of the resulting solution was adjusted to approximately 3 with either 2M sodium acetate or 10% sulfuric acid. The tubes were then placed in a boiling water bath for 20-30 minutes after which the total volume was brought to 5.0 ml with deionized distilled water. The solutions were then centrifuged at 760 xg for 15 minutes to remove the protein precipitate. The absorption of the supernatant at 510 μm was read against a blank using a Beckman DU-2 Spectrophotometer. The iron content was then obtained from a standard curve.

Methionine Analysis. As mentioned previously, since carboxyamido- methylmethionine is not stable to acid hydrolysis it cannot be determined directly by amino acid analysis of the acid hydrolyzed samples. Grundlach et al. (1957) demonstrated, however, that methionine may be quantitated by oxidation of the modified protein with performic acid. This
converts the unreacted methionine to methionine sulfone which is stable to acid hydrolysis. The methionine sulfone which elutes immediately after aspartic acid on the long column of the amino acid analyzer may be determined quantitatively (Grundlach et al. 1957). Thus it becomes possible to determine by difference the initial amount of carboxyamidomethylmethionine. The oxidation procedure employed was basically that of Moore (1962). The performic acid for the reaction was prepared by mixing 9 parts of formic acid with one part hydrogen peroxide and allowing the solution to stand at room temperature for one hour. This solution was then cooled to 0° and 2 ml added to 0.05 to 0.1 µmoles of lyophilized protein in a hydrolysis tube. The reaction was allowed to proceed in an ice bath for 4 hours. Beckman amino acid standard, 2.5 µmoles/ml, (1 ml) oxidized in the same manner, provided a standard value for methionine sulfone. Hydrobromic acid (0.3 ml) was added at the end of 4 hours to quench the reaction. The samples were taken to dryness in a vacuum desiccator using NaOH as a desiccant. A small volume of formic acid was then added and the tubes taken to dryness again. Acid hydrolysis was then carried out for 24 hours. The hydrolysate was analyzed on the long column of the amino acid analyzer.

Localization of the Succinylated or Maleylated Histidine

A sample of the succinylated hemerythrin used in the iodoacetamide experiments was once again succinylated, this time in 6 M guanidinium chloride using a 50 fold molar excess calculated with respect to lysine. The reaction was carried out at room temperature. The other conditions of the succinylation reaction remained the same as those described earlier. A sample of this completely succinylated hemerythrin was
analyzed on the amino acid analyzer.

**Tryptic Digest.** Completely succinylated hemerythrin was made 0.2 M in NH₄HCO₃ and the pH adjusted to 8.1 with NH₄OH. A solution of TPCK-treated trypsin at a concentration of 1 mg/ml was prepared in deionized distilled water. The trypsin was added to the protein at a level corresponding to 0.9% of the protein's weight. The solution was incubated at 31° for three hours. At the end of this time additional trypsin (0.45% by weight) was added and the solution was incubated for an additional hour. The solution was then lyophilized and taken up in 5 ml of deaerated 0.1 M NH₄HCO₃. It was applied to a Sephadex G-50 column (2.5 x 208 cm) and eluted with 0.1 M NH₄HCO₃ at a flow rate of 15-20 ml/hr. Five ml fractions were collected. The A₂₈₀ of each fraction was read on a Hitachi Perkin-Elmer spectrophotometer using a Gilson automatic pipetion unit. The peak tubes from each of the four fractions recovered were subjected to amino acid analysis.

**Chymotryptic Digest.** Fraction TT 2 of the tryptic digest containing residues 50-113 (Klippenstein et al., 1968) was collected and made 0.2 M in NH₄HCO₃. The pH was adjusted to 8.0 with acetic acid. A solution of chymotrypsin (1 mg/ml) was added to the protein at a concentration 0.5% by weight. This solution was incubated at 37° for five hours. At five hours the same amount of chymotrypsin was again added and the incubation was continued for a total of 18 hours. The sample was then lyophilized, taken up in 1 ml of 0.1 M ammonium acetate, pH 5.5, applied to a DEAE-Sephadex column (0.9 x 90 cm) and eluted with a pH 5.5 ammonium acetate gradient (0.1 M to 1M). The tubes were read at 220, 254, and 280 μm. Fifteen fractions were collected and analyzed for their histidine content. Fractions containing the peptides encompassing
residues 69-80 were pooled (D₉₋₁₅).

**Iodoacetamide Modification of Peptide D₉₋₁₅.** A 0.92 μmole sample of the peptide was adjusted to a concentration of 1.5 x 10⁻⁴ M and reacted with iodoacetamide as described previously. The reaction was carried out at 40° for 50 hours. The excess iodoacetamide was removed on a Sephadex G-15 column (1.5 x 90 cm). The sample was eluted with 0.1 M NH₄HCO₃ at a flow rate of 40 ml/hr. One ml samples were collected. The absorbance of the tubes were read at 220, 254 and 280 μm. The peptide fraction was collected, lyophilized and dissolved in 1 ml of deionized distilled water. A 0.1 ml sample was removed and analyzed on the amino acid analyzer. The remainder of the sample was made 0.03 M in HCl and hydrolyzed at 110° C for 12 hours to remove the succinyl groups from the lysines (Klippenstein et al., 1968). After hydrolysis the sample was brought to dryness in the vacuum desiccator and reconstituted in 2 ml of 0.2 M NH₄HCO₃, pH 8.1. It was then digested with trypsin (0.024 mg enzyme at 0 hours and 0.012 mg at 4 hours) for a total of 8 hours. The sample was then lyophilized, dissolved in 0.5 ml of 0.1 M NH₄HCO₃, and placed on the Sephadex G-15 column. It was eluted with 0.1 M NH₄HCO₃ at a flow rate of 7 ml/hr. One ml fractions were collected. The tubes were read at 220, 254 and 280 μm. Each fraction was collected, lyophilized, hydrolyzed and analyzed on the amino acid analyzer.

Peptide maps were also prepared of each fraction. A 20 μl sample in pyridine, acetic acid, water buffer, pH 6.4 (133:4.6:1860 v/v) was applied to Whatman #3 mm chromatography paper and subjected to high voltage electrophoresis (55 V/cm) for 1 hour and 15 minutes. After being allowed to dry, the samples were chromatogrammed in a 1-butanol, acetic acid, water solvent (40:6:15 v/v) for 16 hours. The chromatograms were
dried and developed with 0.1% ninhydrin in 1-butanol:acetone (7:3 v/v).
RESULTS

Methemerythrin Spectra

The visible-ultraviolet spectra of the various methemerythrin derivatives in perchlorate solutions have been reported by Keresztes-Nagy and Klotz (1965a) (Table 1). Since the perchlorate ion has some effect on the iron site, the spectra of the aquo-hydroxy, chloride, fluoride, thiocyanate and azide methemerythrin forms were studied in both unbuffered and buffered (0.1 M Tris acetate, pH 8.0) solutions of the appropriate salt, but in the absence of perchlorate ion. The spectra followed the general pattern reported by Keresztes-Nagy and Klotz but some differences in extinction coefficients were observed in both the unbuffered and buffered solutions. The spectral properties of the aquohydroxy form correspond to those reported by Darnell et al. (1968).

The stability of the spectra to temperature, age, salt concentration and pH was also studied. Each of these factors was shown to have a slight effect. The temperature effect was evaluated by running the spectrum of a metfluoridehemerythrin at 7° and 22° in the water jacketed cell of a Cary Model 15 spectrophotometer (Table 4). The higher temperature caused a decrease in the 317 μ and 473 μ extinction coefficients. The effect of age was evaluated by rerunning the spectra of the fluoride, chloride, thiocyanate, and azide derivatives after a five day interval, the samples having been stored at 5°. All spectra were slightly affected by age but since no constant pattern of change was noted, the differences may have been due to other random factors. The use of 0.1 M versus 1 M
Table 1

Absorption Peaks and Extinction Coefficients of
Methemerythrin Derivatives in the Presence of Perchlorate Ion$^a$

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</tbody>
</table>

$^a$From Keresztes-Nagy and Klotz (1965a).
# Table 2

**Absorption Peaks and Extinction Coefficients of Methemerythrin Derivatives (0.1 M Solutions of the Appropriate Salt)**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$\lambda_{\text{max}}$</th>
<th>$\varepsilon$ (1/cm mole Fe)</th>
<th>$\lambda_{\text{max}}$</th>
<th>$\varepsilon$ (1/cm mole Fe)</th>
<th>$\lambda_{\text{max}}$</th>
<th>$\varepsilon$ (1/cm mole Fe)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N$_3^-$</td>
<td>326</td>
<td>3260</td>
<td>-368</td>
<td>-2178</td>
<td>447</td>
<td>1750</td>
</tr>
<tr>
<td>SCN$^-$</td>
<td>329</td>
<td>3480</td>
<td>-362</td>
<td>-2544</td>
<td>451</td>
<td>2510</td>
</tr>
<tr>
<td>Cl$^-$</td>
<td>331</td>
<td>2941</td>
<td>378</td>
<td>2652</td>
<td>-480</td>
<td>-311</td>
</tr>
<tr>
<td>F$^-$</td>
<td>317</td>
<td>3240</td>
<td>362</td>
<td>2944</td>
<td>-471</td>
<td>-258</td>
</tr>
<tr>
<td>OH,H$_2$O</td>
<td>314</td>
<td>3210</td>
<td>351</td>
<td>2920</td>
<td>-470</td>
<td>-286</td>
</tr>
</tbody>
</table>
### Table 3

Absorption Peaks and Extinction Coefficients of Methemerythrin Derivatives

(0.1 M Solutions of the Appropriate Salt in 0.1 M Tris Acetate Buffer, pH 8.0)

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$\lambda_{max}$</th>
<th>$\varepsilon$ (1/cm mole Fe)</th>
<th>$\lambda_{max}$</th>
<th>$\varepsilon$ (1/cm mole Fe)</th>
<th>$\lambda_{max}$</th>
<th>$\varepsilon$ (1/cm mole Fe)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$N_3$</td>
<td>326</td>
<td>3750</td>
<td>-368</td>
<td>-2500</td>
<td>447</td>
<td>1750</td>
</tr>
<tr>
<td>SCN</td>
<td>328</td>
<td>3314</td>
<td>-367</td>
<td>-2242</td>
<td>448</td>
<td>2420</td>
</tr>
<tr>
<td>Cl</td>
<td>330</td>
<td>3314</td>
<td>379</td>
<td>2845</td>
<td>-485</td>
<td>-425</td>
</tr>
<tr>
<td>F</td>
<td>319</td>
<td>3280</td>
<td>364</td>
<td>2850</td>
<td>-475</td>
<td>-296</td>
</tr>
<tr>
<td>OH, $H_2O$</td>
<td>335</td>
<td>3315</td>
<td>362</td>
<td>3015</td>
<td>-475</td>
<td>-388</td>
</tr>
<tr>
<td>Temp</td>
<td>$\lambda_{\text{max}}$</td>
<td>$\epsilon$ (1/cm mole Fe)</td>
<td>$\lambda_{\text{max}}$</td>
<td>$\epsilon$ (1/cm mole Fe)</td>
<td>$\lambda_{\text{max}}$</td>
<td>$\epsilon$ (1/cm mole Fe)</td>
</tr>
<tr>
<td>------</td>
<td>------------------------</td>
<td>---------------------------</td>
<td>------------------------</td>
<td>---------------------------</td>
<td>------------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>7°</td>
<td>317</td>
<td>3340</td>
<td>363</td>
<td>2830</td>
<td>-473</td>
<td>-262</td>
</tr>
<tr>
<td>22°</td>
<td>317</td>
<td>3250</td>
<td>363</td>
<td>2830</td>
<td>-473</td>
<td>-249</td>
</tr>
</tbody>
</table>
solutions of the salt in 0.1 M Tris acetate buffer, pH 8.0 also produced some variation (Tables 3, 5). Again, there was no consistent pattern noted.

Changes in pH were most crucial with the metaquohydroxyhemery-thrin form (Darnell et al., 1968). pH did, however, affect the other spectra to some extent. Since the acetate ion shows no protein binding affinity, the difference between the spectra in the buffered and unbuffered solutions is presumed to be due to a difference in pH (Table 2, 3). The 0.1 M salt solutions were found to have the following pH values: NaCl - 5.95, KSCN - 5.75, NaN₃ - 9.12 and NaF - 6.85.

This work indicates a number of factors can cause slight variations in the spectra of the various methemerythrin derivatives even when there is no modification of the protein. Therefore, dialysis against 0.1 M solutions of the salts in water was selected and was used in the preparation of all of the various methemerythrin derivatives. A compilation of the data from 18 metazide samples prepared in this manner from the different hemerythrin preparations and evaluated on different spectrophotometers (Cary Model 15, Beckman DU-2 and Perkin-Elmer-Hitachi 124) yielded an average extinction coefficient of 3550 cm⁻¹/mole Fe (standard deviation 163.5) for the 326 μm peak, 2411 cm⁻¹/mole Fe (standard deviation 135.8) for the 368 μm shoulder and 1813 cm⁻¹/mole Fe (standard deviation 228.0) for the 447 μm peak. The apohemerythrin has virtually no absorption in the range of 300-600 μm (Figure 3).

The circular dichroic properties of native metazidehemerythrin were also examined. Hemerythrin exhibits optical activity in the visible region as indicated by its circular dichroism spectrum. The ellipticities in this region for the metazidehemerythrin measured on a Cary Model
Table 5

Absorption Peaks and Extinction Coefficients of Methemerythrin Derivatives

(1.0 M Solutions of the Appropriate Salt in 0.1 M Tris Acetate Buffer, pH 8.0)

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$\lambda_{\text{max}}$</th>
<th>$\varepsilon$ (1/cm mole Fe)</th>
<th>$\lambda_{\text{max}}$</th>
<th>$\varepsilon$ (1/cm mole Fe)</th>
<th>$\lambda_{\text{max}}$</th>
<th>$\varepsilon$ (1/cm mole Fe)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{N}_3^-$</td>
<td>326</td>
<td>3390</td>
<td>-368</td>
<td>-2155</td>
<td>447</td>
<td>1825</td>
</tr>
<tr>
<td>$\text{SCN}^-$</td>
<td>328</td>
<td>3322</td>
<td>-368</td>
<td>-2462</td>
<td>449</td>
<td>2618</td>
</tr>
<tr>
<td>$\text{Cl}^-$</td>
<td>330</td>
<td>2740</td>
<td>381</td>
<td>3420</td>
<td>-485</td>
<td>-540</td>
</tr>
<tr>
<td>$\text{F}^-$</td>
<td>317</td>
<td>3352</td>
<td>362</td>
<td>2980</td>
<td>-470</td>
<td>-260</td>
</tr>
</tbody>
</table>
Figure 3. Spectrum of Apohemerythrin (300-600 μ).
60 spectropolarimeter may be found in Figure 4. This optical activity in the visible region is due to the iron site since it is lost as the site is destroyed (Rill and Klitz, 1970).

The circular dichroism of the metazidehemerythrin in the 210–240 μ range was also measured. It is consistent with a helical protein structure of about 75% helix in agreement with the results reported by Darnall et al. (1969). The ellipticities in this region for the metazidehemerythrin and apohemerythrin can be found in Figure 5. The circular dichroism of the apohemerythrin indicates that much of the helicity is lost when the iron is removed. About one third of the original helix remains. Since the conformation of apohemerythrin is far removed from that of native hemerythrin, it is generally unsuitable for investigations on the active center of the protein.

**Lysine and N-Terminal Glycine Modification Studies**

As was predicted on the basis of its small size and charge properties, methyl acetimidate was the most successful although least reactive amino group modifying agent. A 1000 fold excess of the reagent calculated with respect to lysine was necessary to completely modify the protein in its monomer form. The completely modified protein showed extinction coefficients which were not significantly different from those of the unmodified control (Table 6). Examination of the visible circular dichroism of amidinated sample 1 showed the active center to be intact (Figure 6). All amino groups in these samples were shown to be modified by the fact that no ε-DNP-lysine was obtained after hydrolysis of the modified, dinitrophenylated sample. All 11 lysines could be dinitrophenylated in the unmodified samples. A 300 fold molar excess of methyl-
Figure 4: Circular dichroism of Metazidehemerythrin (320-550 m\textmu).
Figure 5: Circular Dichroism of Metazidehemerythrin and Apohemerythrin (210-240 μ). (— Metazidehemerythrin, --- Apohemerythrin).
ELLIPTICITY x 10^{-3}
### Table 6

**Extinction Coefficients (1/cm mole Fe) of Metazidehemerythrin and Completely Amidinated Metazidehemerythrin Samples**

<table>
<thead>
<tr>
<th>Sample</th>
<th>$326$</th>
<th>$368$</th>
<th>$447$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>3280</td>
<td>2460</td>
<td>1955</td>
</tr>
<tr>
<td>Amidinated Sample 1</td>
<td>3820</td>
<td>2540</td>
<td>1960</td>
</tr>
<tr>
<td>Sample 2</td>
<td>3360</td>
<td>2365</td>
<td>1510</td>
</tr>
<tr>
<td>Amidinated Sample 2</td>
<td>3280</td>
<td>2160</td>
<td>1730</td>
</tr>
</tbody>
</table>
acetimidate was sufficient to modify all but one of the lysines in the protein (Table 7). Thus it appears that most of the lysines in the molecule are readily available for modification. The glycine residue was found to be modified by a 500 fold molar excess of the methyl acetimidate. Modification may well have occurred at lower levels but only this one was analyzed for glycine.

The results from succinylation are not as conclusive as those from amidination in that they gave rise to greater spectral and circular dichroic variation (Table 8, Figure 6). This, however, is not entirely unexpected in that the succinyl group tends to cause conformational distortion of the protein due to its charge properties. As noted with methylacetimidate modification, one lysine residue was difficult to modify. A molar excess of from 4 to 6 with respect to the lysine was sufficient to modify 10 lysines but a 8-10 fold molar excess was necessary to modify all eleven (Table 9).

Maleic anhydride was the least satisfactory modifying agent. With the protein in the monomer form, a three fold molar excess with respect to lysine was sufficient to cause complete modification of the lysines and the N-terminal glycine. The spectrum of the modified protein in the 300-600 μ range showed greater distortion, however, than did that of the succinylated protein. Because of the increased selectivity of this reagent, one would expect that distortion should be about the same or less than that observed in succinylation. The observed difference in extinction coefficients is presumably due to the contribution of the conjugated maleyl lysine to the absorption of the protein at 280 μ. The maleyl amide grouping has been found to have an extinction coefficient of 308 at 280 μ (Butler et al., 1969). When one allows for the contri-
Table 7

Sequential Modification of Lysine Residues

Metazidehemerythrin (Monomer) with Methylacetimidate

<table>
<thead>
<tr>
<th>Molar Excess of Reagent</th>
<th>Modified Lysines</th>
<th>$\epsilon_{326}$ 1/cm mole Fe</th>
<th>$\epsilon_{445}$ 1/cm mole Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>3680</td>
<td>1955</td>
</tr>
<tr>
<td>100</td>
<td>9.75</td>
<td>3895</td>
<td>1765</td>
</tr>
<tr>
<td>300</td>
<td>9.9</td>
<td>3820</td>
<td>1975</td>
</tr>
<tr>
<td>500</td>
<td>10.25</td>
<td>3800</td>
<td>1926</td>
</tr>
<tr>
<td>1000</td>
<td>11.0</td>
<td>3820</td>
<td>1960</td>
</tr>
</tbody>
</table>
Table 8

Extinction Coefficients (1/cm mole Fe) of Metazidehemerythrin and Completely Succinylated Metazidehemerythrin Samples

<table>
<thead>
<tr>
<th></th>
<th>$\lambda_{\text{max}}$ (mu)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>326</td>
</tr>
<tr>
<td>Sample 1</td>
<td>3360</td>
</tr>
<tr>
<td>Succinylated Sample 1</td>
<td>3000</td>
</tr>
<tr>
<td>Sample 2</td>
<td>3690</td>
</tr>
<tr>
<td>Succinylated Sample 2</td>
<td>3020</td>
</tr>
<tr>
<td>Sample 3</td>
<td>3400</td>
</tr>
<tr>
<td>Succinylated Sample 3</td>
<td>3060</td>
</tr>
</tbody>
</table>
Figure 6: Circular Dichroism of Metazidehemerythrin, Completely Succinylated Metazidehemerythrin and Completely Amidinated Metazidehemerythrin (300-500 μm). (--- Metazidehemerythrin, ----- Completely Succinylated Metazidehemerythrin, --- Completely Amidinated Metazidehemerythrin.)
Table 9

Sequential Modification of Lysine Residues
in Metazidehemerythrin (Monomer) with Succinic Anhydride

<table>
<thead>
<tr>
<th>Molar Excess of Reagent</th>
<th>Modified Lysines</th>
<th>ε326 1/cm mole Fe</th>
<th>ε445 1/cm mole Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>3360</td>
<td>1832</td>
</tr>
<tr>
<td>2</td>
<td>7.7</td>
<td>3185</td>
<td>1680</td>
</tr>
<tr>
<td>3</td>
<td>8.6</td>
<td>3310</td>
<td>1718</td>
</tr>
<tr>
<td>4</td>
<td>9.6</td>
<td>3355</td>
<td>1790</td>
</tr>
<tr>
<td>6</td>
<td>9.5</td>
<td>3295</td>
<td>1648</td>
</tr>
<tr>
<td>9</td>
<td>10.8</td>
<td>3240</td>
<td>1520</td>
</tr>
<tr>
<td>12</td>
<td>10.96</td>
<td>3000</td>
<td>1214</td>
</tr>
<tr>
<td>15</td>
<td>11.0</td>
<td>2840</td>
<td>1068</td>
</tr>
</tbody>
</table>
bution of twelve such groups to the 280 m\(\mu\) absorption and estimates the protein concentration on the basis of 280 m\(\mu\) absorption or when a Lowry protein concentration is used, more reliable extinction coefficients are obtained (Table 10). The high 326 m\(\mu\) extinction coefficient based on the protein concentration determined by the Lowry method could well be due to the absorption of the maleyl lysine system at 326 m\(\mu\). The maleate group which has a lower 280 m\(\mu\) absorption than does the maleylamide group does have absorption at 326 m\(\mu\).

Because the products of reaction with 1 and 2 fold molar excesses of maleic anhydride were not analyzed for \(\varepsilon\)-DNP lysine, the resistant lysine noted in succinylation and amidination was not observed. Extinction coefficients of a sample modified with a two fold molar excess of reagent, however, suggest that reaction is not as complete at this level as it is at the 3 fold level (Table II).

These studies, all done on the monomer form of the protein, show conclusively that neither the lysines nor the N-terminal amino acid of hemerythrin are involved in the active center of the protein. They also suggest that one lysine is quite resistant to modification. Studies on the octamer form were also carried out. In both maleylation and amidination, the octamer was more resistant to modification than was the monomer. Extensive maleylation of the octamer tended to leave 0.5-1 residues unmodified at molar excess 10-20 times the lysine concentration while all lysines were modified with a 3 fold molar excess in the monomer. Parallel modification studies by amidination showed modification of 7.8 lysine residues in the monomer and 5.6 residues in the octamer when other conditions of reaction were identical. This suggests that the lysine which is difficult to modify in the monomer may be further protected by
Table 10

Extinction Coefficients (1/cm/mole Fe) of Metazidehemerythin and Completely Maleylated Metazidehemerythin Samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\lambda_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>326</td>
</tr>
<tr>
<td></td>
<td>368</td>
</tr>
<tr>
<td></td>
<td>447</td>
</tr>
<tr>
<td>Maleylated Sample</td>
<td>3544</td>
</tr>
<tr>
<td>(A$_{280}$ Conc.)</td>
<td>2310</td>
</tr>
<tr>
<td></td>
<td>1890</td>
</tr>
<tr>
<td>Maleylated Sample</td>
<td>2890</td>
</tr>
<tr>
<td>(Lowry Conc.)</td>
<td>1860</td>
</tr>
<tr>
<td></td>
<td>1489</td>
</tr>
<tr>
<td>Maleylated Sample</td>
<td>3825</td>
</tr>
<tr>
<td>(Lowry Conc.)</td>
<td>2454</td>
</tr>
<tr>
<td></td>
<td>1954</td>
</tr>
</tbody>
</table>
Table 11

Extinction Coefficients (1/cm mole Fe) of Metazidehemerythin and Partially Maleylated Metazidehemerythin Samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\lambda_{\text{max}}$ (mµ)</th>
<th>326</th>
<th>328</th>
<th>447</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maleylated</td>
<td>3510</td>
<td>2275</td>
<td>1848</td>
<td></td>
</tr>
<tr>
<td>Sample (2X)*</td>
<td>2935</td>
<td>1872</td>
<td>1497</td>
<td></td>
</tr>
<tr>
<td>(A$_{280}$ Conc.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maleylated</td>
<td>2790</td>
<td>1741</td>
<td>1381</td>
<td></td>
</tr>
<tr>
<td>Sample (3X)*</td>
<td>2790</td>
<td>1741</td>
<td>1381</td>
<td></td>
</tr>
<tr>
<td>(A$_{280}$ Conc.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Molar excesses of maleic anhydride calculated with respect to lysine.
subunit interactions and made even more difficult to modify when the protein is in the octamer form. In each case where incomplete modification was observed, either 6, 7 or 10 lysines were modified. Thus it seems that approximately 6-7 lysines are very easy to modify and must be accessible to the reagent in both the monomer and octamer form. Three to four lysines are moderately reactive and one lysine is very unreactive. This last lysine is made even more inaccessible to reagent by subunit interaction.

Hemerythrin Titrations

Full Range Titrations

Excepting arginine, there are 43 titratable groups in hemerythrin. One expects to be able to titrate approximately 24 of these groups within the limited range of pH 4.5 to 10 if all are available (Table 12).

One can use this information to predict the expected titration pattern for the hemerythrin assuming all groups to be available. At the isoelectric point of the protein, the number of acidic groups must equal the number of basic groups. Groups other than the amino acid groups must be considered (i.e. Fe III, N₃, etc.). One may estimate the number of titratable amino acid groups on each side of the isoinic point on the basis of the information in Table 12. Considering the pH ranges over which the groups might be expected to titrate, one may distribute the groups as follows (Table 13). From this information a theoretical titration curve can be constructed (Figure 7). This figure can be compared with the actual plot of the titration data for monomer and octamer (Figure 8). There is a close correlation of the theoretical and actual figures in the basic region from the isoinic point to pH 9.8. This is not the
Table 12

Titration Profile of Hemerythrin (pH 4.5 - 10.0)

<table>
<thead>
<tr>
<th>Residue</th>
<th>No. in Hemerythrin</th>
<th>Approximate pK\textsuperscript{a}</th>
<th>pH Range Titration Observed in Proteins\textsuperscript{b}</th>
<th>No. Expected to Titrater pH 4.5 - 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\alpha)-NH\textsubscript{2} (Gly)</td>
<td>1</td>
<td>7.5</td>
<td>7.8 - 8.3</td>
<td>1.0</td>
</tr>
<tr>
<td>Lys</td>
<td>11</td>
<td>10.0</td>
<td>9.6 - 9.8</td>
<td>5.5</td>
</tr>
<tr>
<td>Tyr</td>
<td>5</td>
<td>9.8</td>
<td>9.1 - 7.13</td>
<td>2.0</td>
</tr>
<tr>
<td>Cys</td>
<td>1</td>
<td>9.5</td>
<td>9.0 - 9.8</td>
<td>0.5</td>
</tr>
<tr>
<td>His</td>
<td>7</td>
<td>6.6</td>
<td>5.5 - 7.4</td>
<td>7.0</td>
</tr>
<tr>
<td>Asp Glu</td>
<td>17</td>
<td>4.6</td>
<td>4.3 - 7.3</td>
<td>8.5</td>
</tr>
<tr>
<td>(\alpha)-COOH (Ile)</td>
<td>1</td>
<td>3.6</td>
<td>1 - 5</td>
<td>-</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Martin (1964)

\textsuperscript{b} Vallee and Wacker (1970)
Table 13

Theoretical Titration of Residues in Hemerythrin Monomer and Hemerythrin Octomer

<table>
<thead>
<tr>
<th>Residue</th>
<th>pH 4.5-PI</th>
<th>pH PI-10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Monomer</td>
<td>Octomer</td>
</tr>
<tr>
<td>COOH (Ile)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Asp, Glu</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>Cys</td>
<td>1 (HMB)</td>
<td></td>
</tr>
<tr>
<td>His</td>
<td>3.4</td>
<td>4.8</td>
</tr>
<tr>
<td>Lys</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>NH₂ (Gly)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Tyr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>22</td>
<td>22.8</td>
</tr>
</tbody>
</table>
Figure 7: Theoretical Titration Curve for Hemerythrin Monomer and Hemerythrin Octamer (--- monomer, --- octamer).
Figure 8: Full Range Titration Curve for Metazidehemerythrin Monomer and Metazidehemerythrin Octamer (--- monomer, --- octamer).
case in the acid titration. One finds that both monomer and octamer
titrates identically down to pH 5.0 but not as theoretically predicted.
Fewer histidines are titrated than would be predicted from the theoreti-
cal curve. These unavailable histidines may be involved in the active
site.

In the actual acid titration of the octamer, denaturation, as
indicated by a time dependent drift in pH, was noted starting at pH 4.19.
The time necessary to establish pH stability increased to a maximum at
pH 3.5. By pH 2.98 the characteristic hemerythrin amber color and thus
the iron had been completely lost. Only two additional groups were
titrated after the loss of the iron. A total of 17.8 groups were
titrated. In the basic region of the titration, pH drift was observed
to commence at pH 10.7-10.8. The amber color of the protein changed
during the titration and precipitation occurred but there was no apparent
loss of the iron. There was also no leveling off in the number of groups
titrated. This, however, is generally to be expected in the basic region
(Nozaki and Tanford, 1967).

The isoinionic point of the monomer (6.7) was lower than that of
the octamer (7.0) as is expected since the cysteine in the monomer had
been modified with p-hydroxymercuribenzoate. Thus, the modified cysteine
residue titrates in the acidic region. In the monomer, denaturation
started at pH 4.7 in the acid region and pH 9.8 in the basic region.
Complete loss of the iron was apparent at pH 3.5. A total of 20.8 groups
were titrated in the acid region. Again, the value in the basic region
of the curve did not level off.

A comparison of the acid titration curve of monomer to that for
octamer shows a distinct difference in the response of the protein to the
addition of acid to groups 4 through 13. It is apparently much easier to titrate these groups in the monomer form than in the octamer. The linear, horizontal portion of the curve indicates that four to six groups are involved in the difference. These groups apparently titrate at pH 4.3 to 4.5 in the monomer and pH 3.5 to 3.8 in the octamer. One may postulate that this difference exists because the environment of the groups in question in the octamer is one which would lower the pK's of these groups. This could be accomplished by an environment of low polarity or an electron withdrawing environment such as would exist if there were salt bridges between the glutamic or aspartic acid residues being titrated and lysine or arginine. The acid denaturation of the octamer starts at about pH 4.2 and that of the monomer at pH 4.7. The iron is completely lost at pH 2.9 in the octamer and pH 3.5 in the monomer. In the acid titration of the octamer 17.8 groups are titrated and 20.8 groups in the monomer. Both of these values differ from the number predicted in Table 11. The number falls short of the number of residues one would expect by 5.0 for the octamer and 1.2 for the monomer. Subunit and/or ligand interactions maintained even in the presence of denaturation might explain the unavailability of these groups.

One may evaluate the basic titration in a similar fashion. On the linear portion of the curve there are four to six groups that are apparently titrating at pH 9.7 - 9.9 in the monomer and pH 10.7 - 10.9 in the octamer. This again suggests an electron donating environment which would be provided by the carboxylate-amino interaction suggested previously. Denaturation begins at pH 10.0 for the octamer and pH 9.0-9.5 for the monomer. The iron color is never completely lost in the basic region implying that none of the basic titrating groups are
essential to the integrity of the iron site.

This data reveals that there are distinct differences in the titration behavior of the monomer and octamer forms of hemerythrin which reflect the state of subunit association. On the basis of the pH region in which these groups titrate the lysine, aspartic acid and glutamic acid residues are most likely those involved in these differences. The data is compatible with a salt bridge type of interaction between these residues. It is also apparent that in both cases histidines are titrating abnormally.

Limited Range Titrations

Nine titrations in the limited pH range of 4.5 - 9 were carried out at 25°C. Since there were some variations in the results of these titrations especially at the lower pH values the results were averaged together to give the curve shown in Figure 9. The protein was found to titrate reversibly in this region. On the basis of this average curve, 2.95 residues are titrated between pH 8.0 and 5.0 and 2.15 between pH 8.0 and 5.5. Since only histidine would normally be expected to titrate in this region, this strongly suggests that only two histidine residues are being titrated. The spectrum of the protein at pH 4.5 was found to be that of native hemerythrin in the 300-600 m\(\mu\) range.

The accuracy of these results was checked by titrating bovine serum albumin under the same physical conditions. An ionic strength of 0.15 \(\mu\) was selected so that the results could be compared with those of Tanford (1955). Two samples were titrated. Again a small variation in results was observed but the correlation with the Tanford data was good, lending credence to the hemerythrin results. Calibration of the micro-
Figure 9: Titration Curves for Metazidehemerythin (pH 4.5-9.0) (5° 15° 25°) (— 25°, --- 15°, ······ 5°).
meter syringes showed that it had lost some precision making it the most probable cause of the slight variance observed. The standard deviation in Z values ranged from a minimum of 0.05 to a maximum of 0.36.

A more accurate estimate of the numbers of histidines titrated may be made on the basis of the heats of ionization of the titratable residues. They may be calculated from the change in pH with temperature at a constant Z value according to the following formula (Nozaki and Tanford (1967)):

\[ Q = 2.303 \frac{RT^2}{Z} \left( \frac{\delta pH}{\delta T} \right)_Z \]

Histidine residues generally are observed to have a heat of ionization of 7 kcal; amino residues (lysine and the N terminal glycine); 11 kcal, and carboxyl residues (aspartic and glutamic acid); 1 kcal (Martin, 1964).

In order to experimentally determine these values in hemerythrin, a number of limited range titrations (pH 4.5 - 9.0) were carried out at 5°, 15° as well as at 25°C. The results of titrations at each temperature were averaged together and are included in Figure 9. The values for \( (\delta pH/\delta T)_Z \) were plotted and the slopes of these lines calculated and used to find maximum and minimum values for the heats of ionization. These calculations resulted in Figure 10 which suggests that between one and two histidines are titrated and that they titrate at a high pH (6.5 - 8.6) (Figure 11).

Although one should be able to calculate the intrinsic pK's of the histidines according to the formula

\[ \log \frac{a}{1 - a} - pH = pK_{int} - 0.868wz \quad a = \frac{Z}{n} \]

in this case no reasonable results were obtained. This formula, however, is only applicable where the number of residues titrated is considerably greater than the one to two titrated in hemerythrin. The precision of
Figure 10: Heats of Ionization for Residues Titrating Between pH 4.5-9.0.
HEAT OF IONIZATION (kcal)
Figure 11: pH Range for Histidine Titration as Determined from Heats of Ionization.
the technique does not allow for cases where Z approaches n as it does in hemerythrin.

On the basis of the high apparent pK of the titratable histidine residues, it appears that they are in an electron withdrawing area of the protein. In terms of primary structure this may implicate residues #77, #73 and #25. Other histidines may be found in such an environment in the tertiary structure of the native molecule.

Titrations in a Denaturing Medium

An attempt was made to titrate additional histidines in a denaturing medium. Guanidinium chloride was the initial choice of the denaturant. With both the protein and blank solutions, however, pH readings were found to be unstable and the amount of titrant consumed in the basic area of the curve considerable. Initially azide solutions were employed but the azide ligand was found to exchange for the chloride ligand present in high concentration from the guanidinium chloride. The spectrum of the hemerythrin was maintained in 3M guanidinium chloride solution but lost in the 6M solution. The same was found to be true when the chloride form of the protein was titrated.

Because of large guanidinium chloride titration correction values, the evaluation of these protein titrations is difficult. In 3M guanidinium chloride solutions, 5.5 groups are apparently titrated in metchloride-hemerythrin and 6.7 in the metazidehemerythrin between pH 5.0 and 8.0. (Figure 12). Allowing for normalization of the amino group and some of the glutamic and aspartic acids, the number of histidines still appears to be approximately 2. The results, however, are approximate because of the large number of titrating groups present in the guanidinium chloride
Figure 12: Titration Curves for Metazidehemerythrin and Metchloride-hemerythrin in 3M Guanidinium Chloride.

--- Metazidehemerythrin

--- Metchloridehemerythrin
and the instability of pH readings encountered in this medium.

Urea solutions were then selected as the denaturant of choice. Three titrations of metazidehemerythrin in 4M urea were carried out from pH 4.5 - 9.0. The results were virtually identical and were congruent with the composite result of the metazidehemerythrin titrations at 25° from pH 8.0 to 6.5 (Figure 13). Below pH 6.5 more groups were apparently titrated in the urea denatured protein than in the native protein. These groups are most probably glutamic and aspartic acid residues. The spectra of the samples before and after titrations were about the same (Table 14). The spectral shift in the metazidehemerythrin shoulder (368 μm) apparently results from some replacement of the azide ligand with water. The circular dichroism of the sample indicates that the conformation and percent of helix is that of the native protein. After removal of the urea by dialysis, a portion of the protein precipitates but the remainder retains a good spectrum. The titration results, precipitation and ligand exchange indicate that the urea does cause some change in the protein's conformation but this change does not affect the helical conformation. These results in conjunction with those of the apohemerythrin studies suggest that the iron may be essential for maintenance of the helix.

Titrations of metaquo and metchloridehemerythrin in urea suggest that these two forms of the protein are much less stable in urea than is the azide form. There is a distinct change in the spectra of these forms of the protein on titration. Since the water and chloride ligands are apparently less tightly bound to the iron (Keresztes-Nagy and Klotz, 1965a) it may be that they stabilize the iron site and its protein ligands less and thereby allow for greater distortion of the protein by urea.

Results of the titration of metazidehemerythrin in 5M and 6M urea
Figure 13: Titration Curves for Metazidehemerythrin in 4M Urea.

--- Metazidehemerythrin (No Urea)

--- Metazidehemerythrin (Urea)
Table 14

Extinction Coefficients (1/cm/mole Fe<sub>cm</sub>) of Metazidehemerythrin in Urea

<table>
<thead>
<tr>
<th>( \lambda_{\text{max}} (\text{m}\mu) )</th>
<th>326</th>
<th>368</th>
<th>447</th>
</tr>
</thead>
<tbody>
<tr>
<td>4M Urea</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 7.2</td>
<td>3655</td>
<td>2496</td>
<td>1690</td>
</tr>
<tr>
<td>pH 5</td>
<td>3473</td>
<td>2725</td>
<td>1445</td>
</tr>
<tr>
<td>After Dialysis</td>
<td>3485</td>
<td>2711</td>
<td>1557</td>
</tr>
<tr>
<td>5M Urea</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 7.2</td>
<td>3372</td>
<td>2443</td>
<td>1504</td>
</tr>
<tr>
<td>pH 5</td>
<td>3500</td>
<td>2845</td>
<td>1364</td>
</tr>
<tr>
<td>After Dialysis</td>
<td>3307</td>
<td>2525</td>
<td>1455</td>
</tr>
<tr>
<td>6M Urea</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 7.2</td>
<td>3384</td>
<td>2486</td>
<td>1512</td>
</tr>
<tr>
<td>pH 5</td>
<td>3096</td>
<td>2640</td>
<td>952</td>
</tr>
<tr>
<td>After Dialysis</td>
<td>3325</td>
<td>2575</td>
<td>1405</td>
</tr>
</tbody>
</table>
are much like those in 4M solutions. In the 6M solution a rather distinct change in spectral shape is evident after the titration. It is possible, however, to dialyze out the urea in both cases and still retain a good portion of native spectrum even without the addition of more azide ion (Table 14).

**Apohemerythrin Titrations**

Two samples of apohemerythrin, one prepared initially from metchloride, the other for metaquohydroxyhemerythrin were examined by circular dichroism and by titration. The circular dichroism studies showed that the two species were similar to each other in terms of percent helicity and that the percent helicity was approximately one third that found in native hemerythrin (Figure 5).

Three titrations were carried out (Figure 14). The curves from two independent titrations of the apohemerythrin derived from metaquohydroxyhemerythrin are virtually congruent. One of these titrations was a simple titration of the protein from its isoelectric point to pH 5, the other involved titration to the point of precipitation, back titration to pH 9.9 and then titration again to pH 5. The third titration was of apohemerythrin derived from metchloridehemerythrin. Nine residues were titrated between pH 8 and 5.5 in the chloride derived apohemerythrin sample and 8.75 in the two aquohydroxy derived apohemerythrin samples. Both these values allow for the titration of all 6.7 histidines, the amino group and some aspartic and glutamic residues. In all three samples the apoprotein precipitated at pH 7.68 - 7.88. It was possible to resolubilize the apoprotein by increasing the pH to 9.7 - 9.9 and to retitrate reversibly to the point of precipitation. However, if the apoprotein was
Figure 14: Titration Curves for Apohemerythrin.

----- Met Aquo Derived Apohemerythrin

----- Met Chloride Derived Apohemerythrin
titrated to pH 5 it was necessary to increase the pH to 10.8 to bring it back into solution. The pH at which precipitation occurs is in the range where the histidines are titrating. The fact that the apoprotein titrated through this region have different solubility properties than the apoprotein which is precipitated at pH 7.8 but not titrated to the lower pH suggests that the titration of histidine is responsible for the change in solubility properties.

Although the titration results of these two different hemerythrins are grossly similar, differences do exist as is evident from Figure 14. These differences suggest that the apohemerythrins are a mixture of different conformational states giving rise to the differences in behavior. This contention is supported by the variance in the 190-240 m\text{\textmu} circular dichroism studies of the two samples (Figure 5). However, the titration results do demonstrate that in a hemerythrin form lacking in iron and with a conformation other than native, the histidines are available for titration. The apohemerythrin still possesses some helix and therefore has some secondary structure. It is relatively unstable, however, and in its isoionic form, precipitates on standing.

**Histidine and Methionine Modification Studies**

The hemerythrin titration studies suggest that one to two histidines are available for titration and thus are not involved in iron binding. This estimation of histidine availability was confirmed by the dinitrophenylation of native and completely succinylated hemerythrin. In each case it was possible to modify 1.5 histidines with the fluoro-2,4-dinitrobenzene. Because of the color of the dinitrophenylated end product, however, it was not feasible to establish the response of the active
site to modification of these residues.

With this preliminary information on the histidines in hemerythrin a kinetic study of the modification of the protein with iodoacetamide was undertaken. The reaction conditions were controlled so that the only amino acid side chains modified appreciably would be those of histidine and methionine. The carboxamidomethylhistidine and carboxamidomethylmethionine derivatives do not of their own accord change the spectral properties of the protein. Also the extent of modification can be determined quantitatively after acid hydrolysis of the protein. Thus this reaction provides a method of correlating the stoichiometry of the modification reaction with the properties of the active site.

The results of the kinetic studies on hemerythrin at pH 8.0 are presented in the following figures. Figure 16 shows the changes in 326 μ and 447 μ extinction coefficients, the iron content and the ellipticity of the succinylated protein as the protein is modified with iodoacetamide; Figure 16, the changes in the visible circular dichroism of selected samples. The base value for the iron in Figure 15 is 0.3 moles of iron per mole of protein instead of the 0.0 moles expected. However, the absorption of these samples at 510 μ in the iron determination was found to be independent of concentration suggesting that the results are due to an artifact other than the iron content. Thus the 0.3 mole base value probably represents no protein-bound iron. These results show that the loss of iron, helicity, visible circular dichroism and UV-visible spectral character occur concurrently between 12 and 32 hours. A first order kinetic plot of the change in each of these properties may be found in Figure 17. In each instance linearity is observed for some portion of the 12-32 hour reaction period. The degree of linearity is poorest for
Figure 15: Changes in Extinction Coefficients (326 and 447 μm), the Ellipticity (222 μm) and the Iron Content During the Reaction of Metazidehemerythrin (Succinylated) with Iodoacetamide.

- ○ ε 326 μm 1/cm mole Fe
- ● ε 447 μm 1/cm mole Fe
- ▲ Ellipticity (222 μm)
- ▼ Iron Content gm atoms/mole monomer
Figure 16: Changes in the Ellipticities (300-500 μ) During the Reaction of Metazidehemerythrin (Succinylated) with Iodoacetamide.

--- 4 hours

----- 18 hours

----- 32 hours
Figure 17: Pseudo First Order Kinetic Plot of the Changes in Extinction Coefficients (326 and 447 μ), Ellipticity (222 μ) and Iron Content During the Reaction of Metazidehemerythin (Succinylated) with Iodoacetamide.

- O ε 326 μ
- 6 ε 447 μ
- ▲ Ellipticity (222 μ)
- ✠ Iron Content
the ellipticity but the fact that this is a property of the whole molecule and not just the active center probably accounts for this result. The sigmoidal curve for ellipticity indicates that the molecular unfolding lags the modification reaction. It should be noted from Figure 17 that very little change of any of the monitored properties occurs in the first 15 hours of reaction or after 35 hours. The first order rate constants for the 15-35 hour period may be found in Table 15.

The results of the analysis for modified histidines and methionines may be found in Figure 18 and 21. It is important to note several points about the modification of histidine. First, the number of recovered histidines in the 0 time sample is low by 0.7 residues. A similar low recovery of histidine was observed in three other analyses of the succinylated hemerythrin, and was also found to be true of a sample completely succinylated at a higher pH (pH 8-9). When the iodoacetamide reaction was carried out at pH 5.5, 0.5 histidines were lost in the 0 time sample. This implies that in the initial succinylation reaction a portion of the histidine becomes modified and the resulting derivative does not appear on amino acid analysis. Since histidines normally react with succinic anhydride to form a very unstable bond, it is presumed that only one histidine is involved and its succinylimido bond is stabilized by some unique environmental properties, i.e. high nucleophilicity. The possibility of reaction with an impurity in the succinic anhydride was eliminated on the basis of an IR spectra of this reagent which revealed no detectable quantities of impurities.

Unusual nucleophilicity might be conferred on a histidine through bifunctional catalysis involving one of its neighbors. On the basis of primary structure this could involve a neighboring unmodified lysine or
Table 15

First Order Rate Constants for Changes Observed in Metazidehemerythrin During Modification with Iodoacetamide

<table>
<thead>
<tr>
<th>Property</th>
<th>R (hr⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extinction coefficient 326 μm</td>
<td>.019</td>
</tr>
<tr>
<td>Extinction coefficient 447 μm</td>
<td>.027</td>
</tr>
<tr>
<td>Iron content</td>
<td>.018+</td>
</tr>
<tr>
<td>Ellipticity (222 μm)</td>
<td>.021</td>
</tr>
</tbody>
</table>
Figure 18: Iodoacetamide Modification of Histidine in Metazidehemerythrin (Succinylated).

- △ Total Modified and Free Histidine
- ★ Histidine
- ○ Total Modified Histidine
- ▲ 3-carboxymethylhistidine
- □ 1-carboxymethylhistidine
- ▣ 1,3-dicarboxymethylhistidine
if the histidine were partially protonated, i.e. one that would have a high pK, the same function could be served by a neighboring glutamic, aspartic or succinyl group. Since almost all the lysines in the molecule have presumably been modified, the former possibility seems unlikely. Since the latter possibility requires protonation of the histidine, the histidine involved may well be one that titrates at a high pH. Still one would expect normal hydrolysis of the succinylimido bond on hydrolysis of the protein and concomitant recovery of the histidine. Apparently hydrolysis does not occur since the histidine is not recovered.

The results using the maleylated hemerythrin are virtually identical to those with succinylated hemerythrin (Figures 19 and 20). Again, the amount of histidine recovered is low but since the maleyl group is similar to the succinyl group in its chemical properties, this is not unexpected.

If one considers the destruction of the active site to begin at 12 hours, then 1.2 histidines in addition to the original 0.7 have been modified at this point. Sequence work suggests that about one half of these 1.2 histidines are residue #34. Residue #82 is also partially modified as is the peptide containing histidines #73 and #77 (Klippenstein - unpublished observations). In the 12-32 hour period of the reaction 2.1 additional histidines are modified. Sequence work suggests that modification at this point is distributed over a number of residues. It thus seems probable that three histidines are not involved in the active site of the molecule, #34 and #82 and the histidine which reacts with succinic anhydride, probably #72 or #73. Once the destruction of the active site begins, the remainder of the histidines apparently can react either at random rates or at rates controlled by their chemical environ-
Figure 19: Changes in Extinction Coefficients (326 and 447 μm) During the Reaction of Metazidehemerythrin (Maleylated) with Iodoacetamide.

- ○ $\varepsilon$ 326 μm (1/cm mole Fe)
- ● $\varepsilon$ 447 μm (1/cm mole Fe)
Figure 20: Iodoacetamide Modification of Histidine in Metazidehemerythrin (Maleylated).

- ▲ Total Modified and Free Histidine
- ★ Histidine
- ○ Total Modified Histidine
- ▲ 3-carboxymethylhistidine
- □ 1-carboxymethylhistidine
- ★ 1,3 dicarboxymethylhistidine
Figure 21: Iodoacetamide Modification of Methionine in Metazidehemerythrin (Succinylated).
RESIDUES METHIONINE

TIME (HOURS)

0

0.5

1.0
ment.

The pseudo first order rate plot for the reaction of histidine and methionine may be found in Figure 22. All the histidines appear to react at the same rate with a rate constant of 0.0049 hr\(^{-1}\). Sequence work, however, shows the rate of modification of the different histidines to differ (Klippenstein, unpublished observations) thus negating the concept that they all react at the same rate.

The results of the methionine analysis show that 0.475 residues have reacted during the first 12 hours of the reaction. The first order plot shows that the rate of methionine reaction (0.0082 hr\(^{-1}\)) is much greater than that of active site destruction (0.0011 hr\(^{-1}\)) over the first twelve hours of reaction. Both these facts suggest that methionine is not involved in the active center. The fact that during the 12-32 hour period the methionine rate of reaction (0.023 hr\(^{-1}\)) approximates that of active site destruction probably means that, at that point, the rate of the change in the conformation of the molecule becomes rate determining for the methionine reaction.

Since the histidine reaction is pH dependent and the methionine reaction is not, the same reaction was carried out at pH 5.5 using a sodium acetate buffer system. As the reaction proceeded, precipitation of the protein occurred. The protein in the supernatant, however, retained the native hemerythrin spectra for up to 16 hours of reaction. At 26 hours most of the protein had precipitated and that portion that remained in solution had lost its native metazidehemerythrin color (Figure 23). Analysis for modified methionine in the soluble protein showed that approximately 0.2 methionine residues had reacted at 16 hours tending to confirm the hypothesis that the methionine is not involved in the active
Figure 22: Pseudo First Order Kinetic Plot for the Iodoacetamide Modification of Histidine and Methionine in Metazide-hemerythrin (Succinylated).

- Histidine
- Methionine
Figure 23: Changes in the Extinction Coefficients (326 and 447 μ
During the Reaction of Metazidehemerythin (Succinylated)
with Iodoacetamide at pH 5.5.

○ ε 326 μ (1/cm mole Fe)

◆ ε 447 μ (1/cm mole Fe)
site. Analysis showed that less than one histidine had been modified at this point. Iron analysis of the 26 hour precipitate which still displayed the native metazidehemerythrin amber color, showed that 2.0 gram atoms of iron were still bound by the protein. Amino acid analysis of the sample showed that 0.76 methionines had been modified but only 0.7 histidines. Samples reacted with iodoacetamide at pH's 6, 6.5 and 7 also showed precipitation. These samples, however, were not analyzed. This information further strengthens the conclusion that methionine is not involved in the iron site but is involved in maintenance of protein conformation. Samples reacted at pH 8.0 in a Tris-cacodylate buffer did not show precipitation on modification of methionine. Therefore, this precipitation seems to be a function of pH. When a sample with 0.35 modified methionines and 0.94 modified histidines was dialyzed against 0.1M Tris cacodylate buffer, pH 6.0, precipitation of the protein occurred. The precipitate again retained its native metazidehemerythrin color.

The reaction of succinylated hemerythrin B with iodoacetamide was also studied. Amino acid composition of this hemerythrin is slightly different from that of a pooled preparation of hemerythrins. The variant composition as determined by Klippenstein (personal communication) may be found in Table 16. The most notable differences in composition are the values for glutamic acid, aspartic acid and histidine. These values reflect the substitution of aspartic acid for glutamic acid at residue #78 and asparagine for histidine at residue #82 (Klippenstein, unpublished observations).

The spectrum (300-600 mu) and A<sub>280</sub> of each sample was determined. Amino acid analysis for the modified histidines and methionines was also carried out after modification with iodoacetamide. On the basis of the
Table 16

Amino Acid Composition of Hemerythrin A and Hemerythrin B (Variant)

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Residues Hr. B</th>
<th>Residues Hr. A</th>
<th>Amino Acid</th>
<th>Residues Hr. B</th>
<th>Residues Hr. A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys</td>
<td>11</td>
<td>11</td>
<td>Gly</td>
<td>7.0</td>
<td>6.6</td>
</tr>
<tr>
<td>His</td>
<td>6.1</td>
<td>6.8</td>
<td>Ala</td>
<td>5.8</td>
<td>5.2</td>
</tr>
<tr>
<td>Arg</td>
<td>2.9</td>
<td>2.9</td>
<td>Val</td>
<td>4.0</td>
<td>3.9</td>
</tr>
<tr>
<td>Asp</td>
<td>19.1</td>
<td>17.0</td>
<td>Met</td>
<td>.83</td>
<td>.84</td>
</tr>
<tr>
<td>Thr</td>
<td>4.1</td>
<td>4.4</td>
<td>Ile</td>
<td>8.9</td>
<td>8.2</td>
</tr>
<tr>
<td>Ser</td>
<td>3.3</td>
<td>3.8</td>
<td>Leu</td>
<td>8.4</td>
<td>8.0</td>
</tr>
<tr>
<td>Glu</td>
<td>9.0</td>
<td>10.1</td>
<td>Tyr</td>
<td>4.8</td>
<td>4.9</td>
</tr>
<tr>
<td>Pro</td>
<td>4.0</td>
<td>4.1</td>
<td>Phe</td>
<td>8.8</td>
<td>9.2</td>
</tr>
</tbody>
</table>
spectral extinction coefficients, destruction of the active site begins at 9 hours and is complete by 28 hours of reaction (Figure 24). Although the active site destruction apparently starts at an earlier time than observed in the previous study of pooled hemerythrin, the pseudo first order rate of active site destruction as reflected in the change in extinction coefficients is quite similar (0.0228 hr\(^{-1}\) \(\varepsilon_{326}\), 0.0248 hr\(^{-1}\) \(\varepsilon_{447}\)).

Results of the analysis for histidine and modified histidine are found in Figure 25. Again the initial 0 hour sample is low by 0.7 residues of histidine. This tends to confirm the supposition that there is rapid reaction of one histidine with succinic anhydride. Since this reaction occurs with the variant it demonstrates that this residue cannot be #82.

If 9 to 28 hours is selected as the time of the active site destruction reaction, then one finds that 0.8 histidine residues react before destruction begins. This is in contrast with 1.2 residues observed in the earlier study of pooled hemerythrin. This difference can be accounted for by the reaction of 0.4 residues of histidine #82 in the pooled preparation. During the time of active site destruction, 2.1 residues of histidine react as compared to the same value with the pooled preparation. The first order rate plot of the histidine disappearance in the period of active site destruction has a rate constant (0.0052 hr\(^{-1}\)) quite close to that observed in the preceding study (Figure 26).

Again the results on the extent of methionine reaction (Figure 27) suggest that it is not involved in the active center. At 0.9 hours, .35 methionine residues, about one third of the total, had reacted. The initial pseudo first order rate of reaction is 0.0075 hr\(^{-1}\). This rate
Figure 24: Changes in the Extinction Coefficients (326 and 447 μm
During the Reaction of Metazidehemerythrin (Succinylated)
with Iodoacetamide.

○ $\varepsilon$ 326 μm 1/cm mole Fe

○ $\varepsilon$ 447 μm 1/cm mole Fe
Figure 25: Iodoacetamide Modification of Histidine in Metazidehemerythrin B (Succinylated).

- △ Total Modified and Free Histidine
- ● Histidine
- ○ Total Modified Histidine
- ▲ 3-carboxymethylhistidine
- □ 1-carboxymethylhistidine
- ◇ 1,3-carboxymethylhistidine
Figure 26: Pseudo First Order Kinetic Plot for the Iodoacetamide Modification of Histidine and Methionine in Metazide-hemerythrin B (Succinylated).

○ Histidine
▲ Methionine
Figure 27: Iodoacetamide Modification of Methionine in Metazidehemery-thrin B (Succinylated).
changes to 0.018 hr\(^{-1}\) as the active site begins to react (Figure 26). These values correlate well with those observed previously and lead to the same interpretation, i.e. that the methionine rate of reaction changes as the helix begins to unwind since at this point this becomes the limiting factor in the reaction. The high initial rate of reaction, however, eliminates methionine as a potential ligand.

The data on the histidines when taken in conjunction with the previous study of pooled hemerythrin and the sequence analysis work indicates that three histidine residues are not involved in the active site and can be modified by iodoacetamide with no change in the character of the hemerythrin. These residues may include the one that is partially succinylated, probably #73 or 77, residue #34 which has been shown to be modified to about 0.6 residues and residue #82, which has been shown to be partially modified in the pooled hemerythrin and which can account for the difference between the number of initially modified residues in the pooled preparation and the variant studies. Methionine is clearly not involved in the active center.

**Localization of the Succinylated Histidine**

On the basis of the nature of the succinylation reaction the histidine most likely to form a stable succinylimido bond or maleylimido bond would be a partially protonated histidine. Since the modification reaction was carried out at pH 8.0, this histidine is most likely one demonstrated to have a high pK\(_{\text{int}}\) in the titration studies. The nature of the primary structure of the protein would suggest that residue #77 with neighboring glutamic acid residues could well have a high pK\(_{\text{int}}\). Residue #78 is a site of amino acid interchange in hemerythrin B involving the substitution
of an aspartic acid for a glutamic acid. However this should not greatly affect the pK_{int} of the histidine. The histidines at positions #25 and #73 with neighboring glutamic acids and lysines are also possible candidates since in the succinylated protein the lysines would most likely be succinylated thus having the effect of a second acid residue. These two histidines, however, would be less likely to titrate at a high pK_{int} unless the tertiary structure of the protein caused increased nucleophilicity of the histidine.

In an attempt to localize the succinylated or maleylated histidine, metazidehemerythrin was completely succinylated in guanidinium chloride (6M) at a level 50 times its lysine content. It was then digested with trypsin. Amino acid analysis of the succinylated protein showed a recovery of only 5.7 of the 6.7 histidines. Fraction TT 2 of the tryptic digest gave a recovery of only 3.9 of the 4.7 histidines expected. This fraction contains the C terminal portion of the protein, residues 50-113 (Klippenstein et al., 1968). Fraction TT 3 containing residues #25 and 34 (Klippenstein et al., 1968) showed its normal complement of histidine on amino acid analysis. Thus residue #25 may be eliminated as the succinylated histidine.

Fraction TT 2 was further fragmented with chymotrypsin and purified on a DEAE-Sephadex column. Fifteen fractions were collected and analyzed. Fractions 9, 10, 12, 13, 14 and 15 (peptide D_{9-15}) contained peptides encompassing histidines #73 and #77. These fractions were pooled. On analysis, only 1.45 histidines of the two expected were recovered. A fraction of this histidine can be accounted for by some histidine #82 resulting from incomplete cleavage at phenylalanine #80. Other histidine containing peptides from TT 2 were found to have their expected histidine
content. It thus appears certain that the histidine modified by succinylation is either #73 or #77.

Isolation of the succinylated histidine beyond this point became difficult because of the chemical nature of the peptide. Papain and subtilisin caused no cleavage of the peptide between the two histidines. In another approach the peptide was modified with iodoacetamide for 50 hours to modify the free histidine. After modification the peptide was desalted on a Sephadex G-15 column and subjected to amino acid analysis. It was found to have a total of 1.25 histidines, 90% of which had been modified. The value for total histidine is lower than that of the initial peptide in that further purification of the peptide resulted from the Sephadex G-15 column used to remove the excess iodoacetamide. The modified histidine was found as 3-carboxymethylhistidine and 1,3-dicarboxymethylhistidine. The modified peptide was then hydrolyzed with 0.03M hydrochloric acid to remove the succinyl groups from the lysines (Klippenstein et al., 1968) and then digested with trypsin to cleave the peptide at a point between the two histidine residues. The digest was run through a Sephadex G-15 column; six fractions were collected and analyzed. Peptide maps were also prepared of each fraction. The results of these analyses plus those of the original peptide and the modified peptide can be found in Tables 17 and 18.

One would expect the products of the acid and tryptic digests to be aspartic acid and tyrosine or the dipeptide Tyr-Asp (#70, 71), the tripeptide Glu-His-Lys (#72-74), free lysine (#75) and the pentapeptide Glu-His-Glu-Gly/Thr-Phe (#76-80). Several facts are indicated by the data (Tables 17 and 18). It is possible to isolate the intact peptide (A-T-G) after the dilute acid, tryptic digest ion with some enrichment in 1,3-
Table 17

Amino Acid Composition of the D₉₋₁₅ Peptide, the Modified D₉₋₁₅ Peptide and Its Dilute Acid, Tryptic Digest Fragments

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
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<tbody>
<tr>
<td>Lys</td>
<td>2.3</td>
<td>2.1</td>
<td>2.0</td>
<td>1.2</td>
<td>1.5</td>
<td>0.4</td>
<td>trace</td>
<td>trace</td>
</tr>
<tr>
<td>His</td>
<td>1.4</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3-CMH*</td>
<td>-</td>
<td>0.6</td>
<td>0.6</td>
<td>0.4</td>
<td>0.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1,3-CMH**</td>
<td>-</td>
<td>0.5</td>
<td>0.9</td>
<td>0.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Asp</td>
<td>1.3</td>
<td>0.9</td>
<td>0.4</td>
<td>0.3</td>
<td>1.0</td>
<td>0.4</td>
<td>trace</td>
<td>trace</td>
</tr>
<tr>
<td>Thr</td>
<td>0.4</td>
<td>0.4</td>
<td>0.5</td>
<td>0.1</td>
<td>0.1</td>
<td>0.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glu</td>
<td>2.7</td>
<td>2.5</td>
<td>3.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.3</td>
<td>trace</td>
<td>+</td>
</tr>
<tr>
<td>Gly</td>
<td>0.9</td>
<td>0.9</td>
<td>0.7</td>
<td>0.4</td>
<td>0.5</td>
<td>1.2</td>
<td>trace</td>
<td>trace</td>
</tr>
<tr>
<td>Tyr</td>
<td>0.8</td>
<td>0.7</td>
<td>0.2</td>
<td>0.1</td>
<td>1.0</td>
<td>-</td>
<td>trace</td>
<td>-</td>
</tr>
<tr>
<td>Phe</td>
<td>1.2</td>
<td>1.2</td>
<td>1.0</td>
<td>0.2</td>
<td>-</td>
<td>1.0</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* 3-CMH = 3-carboxymethylhistidine

** 1,3-CMH = 1,3-dicarboxymethylhistidine
Table 18

Electrophoretic Mobility and Proposed Composition of the Dilute Acid, Tryptic Digest Fragments of the Dg_i Peptide

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Acid Peptide</th>
<th>Basic Peptides</th>
<th>Amino Acids</th>
<th>Composition of the Major Peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-T-G₁</td>
<td>4</td>
<td></td>
<td></td>
<td>Glu-His-Lys-Lys-Glu-His-Glu-Gly-Phe</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3-CMH*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1,3-CMH**</td>
</tr>
<tr>
<td>A-T-G₂</td>
<td></td>
<td>5</td>
<td></td>
<td>Glu-His-Lys</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3-CMH</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1,3-CMH</td>
</tr>
<tr>
<td>A-T-G₃</td>
<td>1</td>
<td>1</td>
<td></td>
<td>Glu-His-Lys and Asp-Tyr</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3-CMH</td>
</tr>
<tr>
<td>A-T-G₄</td>
<td>2</td>
<td></td>
<td>Acidic</td>
<td>Glu-Gly(Thr)-Phe</td>
</tr>
<tr>
<td>A-T-G₅</td>
<td></td>
<td></td>
<td>Acidic Basic</td>
<td>Amino Acids</td>
</tr>
<tr>
<td>A-T-G₆</td>
<td></td>
<td></td>
<td>Acidic Basic</td>
<td>Amino Acids</td>
</tr>
</tbody>
</table>

* 3-CMH = 3-carboxymethylhistidine
** 1,3-CMH = 1,3-dicarboxymethylhistidine
*** Succinylated histidine
dicarboxymethylhistidine. This suggests some preferential cleavage of the chains with 3-carboxymethylhistidine. The aspartic acid and tyrosine have essentially been removed. The tripeptide Glu-His-Lys is also isolated as is the tripeptide Glu-Gly-Phe. The histidine in the Glu-His-Lys tripeptide is almost completely modified. Both 1,3-dicarboxymethylhistidine and 3-carboxymethylhistidine are found, but the 3-carboxymethylhistidine predominates, supporting the hypothesis on preferential cleavage of chains containing 3-carboxymethylhistidine. The pentapeptide Glu-His-Glu-Gly-Phe is not detected. This leads to the conclusion that degradation of this peptide has occurred yielding the tripeptide Glu-Gly-Phe. Free glutamic acid can be detected in fraction A-T-G6 which could account for glutamic acid #76. No histidine or carboxymethylhistidine appears in any of these later fractions. Thus it must be that histidine #77 is succinylated and degraded to a new end product. The end product of this reaction was not detected on the amino acid analyzer but it must be one that makes the peptide chain labile to cleavage at position 77 yielding the tripeptide Glu-Gly-Phe. Since such fragmentation would not occur if the histidine existed as free histidine or carboxymethylhistidine, this occurrence further strengthens the conclusion that histidine #77 is the one modified with succinic anhydride or maleic anhydride. Since this modification causes no change in the active site properties of the protein histidine, #77 cannot be involved in the active center.
DISCUSSION

The preceding studies on hemerythrin have been both qualitative and quantitative in nature, being concerned with ascertaining whether lysine, methionine and histidine were involved in the active center of hemerythrin and with estimating the number of these residues so involved. In fulfillment of this intent the preceding data has shown that lysine, glycine and methionine are not involved. In each case this contention is based on the successful chemical modification of the residues without concomitant destruction of the protein's active center as reflected in its spectral and circular dichroic properties.

Although Fan and York (1969) were able to modify 11 amino groups with trinitrobenzenesulfonic acid without a change in the 330 μm extinction coefficient or loss of iron, this did not eliminate all amino groups from active site contention as they claimed since there are a total of twelve amino groups in the protein, 11 lysines and the N-terminal glycine. Garbett et al. (1971b) were not able to modify more than 9 amino groups in the metazo, methydroxy and metazide forms of the protein without causing precipitation and loss of the iron. The data presented here, however, demonstrates that it is possible to modify the eleven lysines and the N-terminal glycine in metazidehemerythrin with methylacetimidate, succinic anhydride and maleic anhydride. In each case the UV-visible spectrum (300-600 μm) of the metazidehemerythrin is not significantly changed nor is the circular dichroism (300-550 μm) of the amidinated and succinylated samples. The difficulty encountered by Garbett et al. (1971b) with succinic anhydride modification may have been due to the fact that
they started with the octameric form of the protein. Even though succinylation causes dissociation of the protein to monomer the initial use of octamer does seem to reduce the reactivity of 3-4 lysines. Even in the monomer form of the protein there is one lysine which can be modified only with a large excess of reagent.

Approximately 40-50% of the methionine can be modified with iodoacetamide at pH 8.0 without appreciably affecting the extinction coefficients of metazidehemerythrin at 326 m\(\mu\) and 447 m\(\mu\), the ellipticity at 222 m\(\mu\), and the iron content of the protein. Destruction of the active center occurs beyond this point, but is due to the modification of histidine and not the reaction of methionine. When the histidine modification is restricted at pH 5.5 it is possible to modify -70% of the methionine and still retain all the protein iron. However, precipitation of the protein does occur when the methionine is modified at this pH. On the basis of this data the methionine can be eliminated as an iron ligand.

Some of the histidine residues apparently are involved in the active center. This fact was suggested by Fan and York (1969) when they were able to modify three histidine residues in oxyhemerythrin monomer and seven residues in apohemerythrin using 5-diazo-1-H tetrazole. The results of Fan and York are confirmed by these studies. Two of the three histidines not involved can be detected by both titration and modification and the third only by modification. This may be due to the fact that the third residue lies in an apolar region of the protein or may reflect the fact that the titration studies were done primarily on hemerythrin octamer and the modification studies on hemerythrin monomer. Fan and York (1968) report that one histidine is much more reactive in monomer than it is in octamer. In titration studies on the hemerythrin monomer, however, little additional
titratable histidine could be detected.

Modification studies of pure hemerythrin B as compared with pooled samples containing both hemerythrin A and hemerythrin B demonstrate that residue #82, the histidine that is replaced by asparagine in hemerythrin B, is modified with iodoacetamide. Partial modification of this residue has also been observed in sequence work on the modified pooled protein by Klippenstein (unpublished observations). He has also demonstrated that residue #34 is modified with iodoacetamide. The third modified residue is apparently #77. This residue is most likely one which titrates at the high pH observed in the titration studies. Histidine residue 77 does not react with iodoacetamide to any appreciable extent (Klippenstein, unpublished observations) because it is modified during the preliminary modification with succinic anhydride or maleic anhydride and then is destroyed during subsequent treatment of the protein. Iodoacetamide modification of the succinylated peptide containing this residue as well as histidine #73 has demonstrated that most of the modification occurs on residue #73 designating #77 as the one modified with succinic anhydride. The peptide containing this residue is degraded by dilute acid, tryptic digestion. Cleavage occurs at position #77 supporting the hypothesis that this residue has been modified by succinic anhydride yielding an end product that is susceptible to cleavage. Such a break in the peptide chain would not occur if histidine or carboxymethylhistidine were found in position #77. Residue #77, thus, is not involved in the active center of the protein. Whether or not the remaining four histidines are actually involved in the active center as suggested by Fan and York (1969) remains to be determined. However, since reaction of these histidines (#'s 25, 54, 73, 101) causes destruction of the active site, at least one and possibly
all four do serve as iron ligands.

In the course of this work a number of facts have been revealed relating to the tertiary and quarternary structure of the protein and the amino acid residues studied. Garbett et al. (1971b, 1971c) have noted that in metaquo and methyldroxyhemerythrin perchlorate ion binding limits the reactivity of two lysines presumably located near the iron site. They did not observe this effect when using metazidehemerythrin. In all studies reported here differential reactivity of the lysines was observed. In the metazidehemerythrin monomer one lysine was found to be very difficult to modify. It should be noted that in all cases the protein was in borate buffer. Borate does have some tendency to bind to the protein and could possibly be responsible for the poor reactivity of this lysine. In metazidehemerythrin octomer three lysines were relatively difficult to modify and a fourth very difficult to modify even with a large excess of the modifying reagent. This fourth lysine is presumably the one which is unreactive in the monomer. It is also probably the residue not reacted by Fan and York (1969) in their studies using trinitrobenzenesulfonic acid.

Differences involving the behavior of lysines in the monomer and octamer were also revealed in the titration studies of both forms of metazidehemerythrin. Approximately four lysines titrate about one pH unit lower in the monomer than they do in the octamer. A complementary difference is noted with the glutamic and aspartic acid residues. In this instance four to six groups titrate 0.5 - 1 pH units higher in the monomer than they do in the octamer. This pattern of difference suggests that some glutamic acid and/or aspartic acid-lysine salt bridge interactions may occur in the octamer form of the protein. It was also noted in the titration studies that the loss of iron occurs 0.6 pH units higher in the
monomer than it does on the octamer suggesting that some protection may be afforded the iron site by octomeric interactions. This hypothesis is supported by the fact that it is virtually impossible to prepare the apohemerythrin form of the protein according to the procedure described by Fan and York (1971) when the octomer form of the protein is used.

Methionine, when it is modified extensively, tends to cause precipitation of the protein at pH values below pH 7.0. This was also noted in earlier methionine modification studies with iodoacetate and sodium periodate (Klippenstein, unpublished observations). Thus, although not involved in the active center, methionine does seem to be related to the maintenance of the protein's native conformation since precipitation occurs with its modification at pH's where unmodified hemerythrin is soluble. The iron also seems to be essential in this respect since the apohemerythrin contains only about one third the helix of the native form and also precipitates on standing at 5° and at room temperature.

Studies on the lysines, glycine, methionine and histidines in hemerythrin have been presented here. Some of the histidines have been identified as being essential to the active center of the protein, four probably serving as ligands for the iron. Although lysine, glycine and methionine have been eliminated as iron ligands, they appear to be involved in maintaining the tertiary and quarternary structure of the protein. The lysines are involved in subunit interactions; the methionine in maintenance of the tertiary conformation. The glycine appears to have no particular role in the organization of the protein at the secondary, tertiary or quarternary level.
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