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Ethoxyformylation and Photooxidation of Histidines in Transferrins†

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ABSTRACT: The chemical reactivity of histidines in ovotransferrin and human serum transferrin was studied utilizing two different reactions. Upon dye-sensitized photooxidation of ovotransferrin and ethoxyformylation of human serum transferrin and ovotransferrin, losses in histidine and iron-binding activity were observed. All of the histidines in both apoproteins could be ethoxyformylated by the use of 170 to 400 molar excesses of reagent resulting in complete loss in activity. The histidines of human serum transferrin showed a greater reactivity toward the reagent than did those of ovotransferrin. The binding of each iron protected two histidines from ethoxyformylation, and in both cases the proteins remained completely active. First-order losses in histidine and iron-binding activity were observed when ovotransferrin was irradiated in the presence of methylene blue. Comparison of the first-order rates indicates the loss of two histidines per binding site accounts for the inactivation of the protein. However, iron binding did not protect ovotransferrin from photoinactivation as expected. Evidence from both modification techniques indicates: (1) Histidines are essential for iron-binding activity. (2) There are two essential histidines in each binding site. The advantages of using two modification reactions, ethoxyformylation and photooxidation, in the study of the functional role of histidines in proteins are demonstrated in this work.

The transferrins are a class of vertebrate glycoproteins which chelate iron in two specific sites on each molecule. Chemical modification is a technique that has been very useful in studying the iron-binding sites of the transferrins. *N*-Acetylimidazole (Komatsu and Feeney, 1967), tetranitromethane (Tsao et al., 1974; Line et al., 1967), and iodine (Komatsu and Feeney, 1967; Azari and Feeney, 1961; Phillips and Azari, 1972) modifications have implicated tyrosine residues as essential for iron binding. Spectroscopic studies have shown that there is at least one nitrogen coordinated to the metal in the binding center and perhaps as many as three are involved (Spartalian et al., 1973; Windle et al., 1963). EPR[1] studies on copper-transferrin complexes indicate that there are as many as four nitrogens available for coordination in the iron-binding site (Aasa and Aisen, 1968).

A number of nitrogen-containing amino acid side chains have been excluded as essential for the activity of the transferrins. Reductive alkylation and trinitrophenylation of human serum transferrin (Means and Feeney, 1968; Zschocke et al., 1972) as well as succinylation and acetylation of chicken ovotransferrin (Buttkus et al., 1965) indicate that amino groups are not directly essential for activity but may be important to the conformational integrity of the protein. Tryptophan is considered nonessential from the chemical-modification studies of human serum transferrin with 2-hydroxy-5-nitrobenzyl bromide (Ford-Hutchinson and Perkins, 1972).

Chemical studies on human serum transferrin with bromoacetate, 5-diazonium-1*H*-tetrazole (Line et al., 1967) and, more recently, ethoxyformic anhydride (Krysteva et al., 1975) have implicated histidines in the metal-binding center. The conclusion that there are two histidines per binding site, derived from the bromoacetate reaction data, is questionable, since the iron-binding activity is rapidly lost only after more than ten residues have been carboxymethylated; yet, the apparent random reaction of histidines shows no such cooperative effect.

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[1] Abbreviations used are: Fe-NTA, iron nitrilotriacetate; EDTA, (ethylenedinitrilo)tetraacetic acid; EPR, electron paramagnetic resonance.

Such evidence may also indicate conformational changes leading to the loss of activity. In addition, the reactions with bromoacetate were found to be nonspecific, amino groups being alkylated as well as histidines. Ethoxyformylation of human serum transferrin and lactoferrin (Krysteva et al., 1975) gave variable results, indicating 1.5 and 0.5 histidines per iron site, respectively.

The original intent of this study was to prove or disprove qualitatively that histidines are essential for iron-binding activity in the transferrins by utilizing two different modification reactions, ethoxyformylation and dye-sensitized photooxidation. Secondly, if histidines were found to be essential, the intent was to determine the number of essential nitrogen ligands, a point of some confusion, by utilizing kinetic data from the modification reactions and protection experiments. During the course of the investigation, it became obvious that each of these two reagents, although reacting with histidines in the same protein systems, exhibited different properties, each contributing unique advantages to the overall experimental design.

Experimental Procedure

Materials

Chicken egg white ovotransferrin was isolated as previously described (Feeney and Komatsu, 1966) and modified to include a final purification step of gel filtration on Sephadex G-100. Ovotransferrin was used in the majority of experiments because it was readily available in a highly purified form. Human serum transferrin was purchased from Sigma Chemical Co. Methylene blue (K & K Laboratories) and ethoxyformic anhydride (Aldrich Chemical Co.) were reagent grade and were used without further purification.

Methods

Preparation of Apotransferrin and Diferric Transferrin. Iron-free ovotransferrin and human serum transferrin were prepared by dialysis first against 0.10 M acetate–0.01 M EDTA at pH 4.5 and then against deionized water prior to lyophilization. All buffers used with the apoovotransferrin modifications were extracted with dithizone–CCl_4, and all glassware and pipettes used were rinsed with concentrated nitric acid and glass distilled water. The diferric form of the protein was prepared by adding 2.5 equiv of an Fe-NTA solution, pH 4.0, to ovotransferrin in 0.05 M NH_4HCO_3. Excess iron was removed by gel filtration with Sephadex G-25 equilibrated with the same buffer. The digallium–ovotransferrin complex was prepared by the addition of a gallium(III) nitrate solution, pH 2, to the protein in buffer. Extinction values of $A_{280}^{1\%} = 11.1$ for apoovotransferrin and $A_{470}^{1\%} = 0.62$ for diferric ovotransferrin were used in the calculations (Warner and Weber, 1951).

Modification of Ovotransferrin with Ethoxyformic Anhydride. Typically, aliquots of ethoxyformic anhydride were added directly to a stirred 3.90 μM ovotransferrin solution in 0.05 M phosphate buffer, pH 7.35, at room temperature. The reagent was added to a final concentration of 0.5–3.0 μL/mL. Stirring was continued for several minutes to bring the ethoxyformic anhydride into solution. The extent of ethoxyformyl/histidine formation was determined by an increase in A_{242} of the reaction solution ($E_{242} = 3.2 \times 10^3$ M^{-1} cm^{-1}) (Ovàdi et al., 1967). In those experiments where iron-binding activity determinations were made, 50 μM protein, 6 mM ethoxyformic anhydride solutions were used. Aliquots removed from the reaction mixture were subjected to gel filtration on Sephadex G-25 to remove the reagent. The activity was then determined by addition of an excess of a Fe-NTA solution and measuring the increase in A_{470} values relative to an unmodified control. The reaction was reversed by incubating these reagent-free solutions in 0.10 M hydroxylamine in the same pH 7.35 buffer. After 20 min, the hydroxylamine was removed by gel filtration with Sephadex G-25 and the activity was determined as described above.

Photooxidation of Ovotransferrin. A Gilson differential respirometer equipped with 40-W incandescent flood lamps approximately 11 cm from the reaction vessel was the light source. In a typical experiment, 4.0 mL of a solution containing 6.50 μM ovotransferrin, in 0.05 M phosphate buffer, pH 7.35, and 0.05% methylene blue was irradiated at 22 °C. In the dark, the reaction was halted by passing the reaction solution through a Sephadex G-25 column (12 × 1.2 cm) to separate the dye from the modified protein. The eluate ovotransferrin was collected in 5-mL fractions, then dialyzed first vs. 0.10 M acetate–0.01 M EDTA at pH 4.5, followed by H_2O prior to lyophilization. Control experiments included incubation in the absence of light as well as irradiation experiments in which the dye was omitted.

Amino Acid Analysis. Losses in tyrosine and histidine during the course of the photooxidation reaction were followed by amino acid analysis. Two to three milligrams of the lyophilized modified protein was sealed in an evacuated tube with 2 mL of 6 N HCl. The samples were hydrolyzed for 22 h at 110 °C. Amino acid analysis was performed on a Technicon Auto Analyzer. Losses in tyrosine and histidine were calculated relative to the phenylalanine peak.

Activity Determination. The loss in iron-binding activity upon photooxidation was determined by a spectrophotometric titration at 470 nm. Metal ions were removed from their ovotransferrin complexes by dialysis vs. 0.01 M EDTA at low pH prior to the activity determination. A 0.85-mL portion of a 10 mg/mL solution of modified ovotransferrin in 0.05 M NH_4HCO_3 at pH 7.8 was titrated with a standardized Fe-NTA solution (standardization procedure described by Harris and Aisen, 1975). A plot of the A_{470} values vs. the amount of iron added permitted calculation of an equivalence point. The concentration of protein was determined by the Lowry technique (Lowry et al., 1951).

Results

Ethoxyformylation of Apoovotransferrin. Initial experiments were performed in order to modify a maximum number of histidine residues in the apoovotransferrin. As the reagent concentration was increased from 3 to 18 mM, the maximum number of histidines modified increased from 9.5 to a maximum of 13.3 residues (Figure 1). This maximum number is in good agreement with the total number of histidines in the molecule (Williams, 1962). Occasionally, more concentrated solutions of apoovotransferrin were used so that the iron-binding activity could be monitored. These experiments indicated that when 40–50% of the histidines were modified, little iron-binding activity remained.

Ethoxyformylation of Diferric Ovotransferrin. Diferric ovotransferrin samples were also exposed to ethoxyformic anhydride in order to modify a maximum number of histidine residues. Figure 1 shows the results when a 360 molar excess of ethoxyformic anhydride was used. The iron-binding activity was not lost, since reactions using more concentrated diferric ovotransferrin solutions showed no decrease in absorbance at 470 nm with the same excess of reagent. A maximum of 9.3 residues were modified in diferric ovotransferrin, while 13.3 histidines were ethoxyformylated in the apoprotein under the

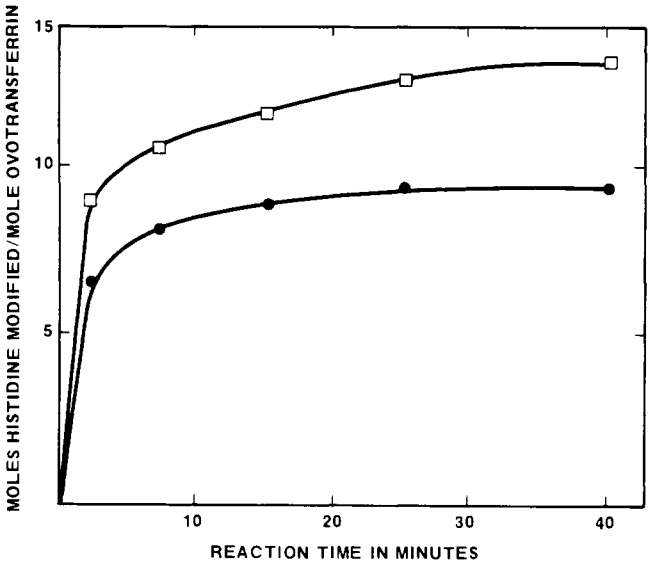

FIGURE 1: Relative reactivities of apoovotransferrin (□, 3.90 μM) and diferric ovotransferrin (●, 3.90 μM) toward ethoxyformic anhydride (18 mM).

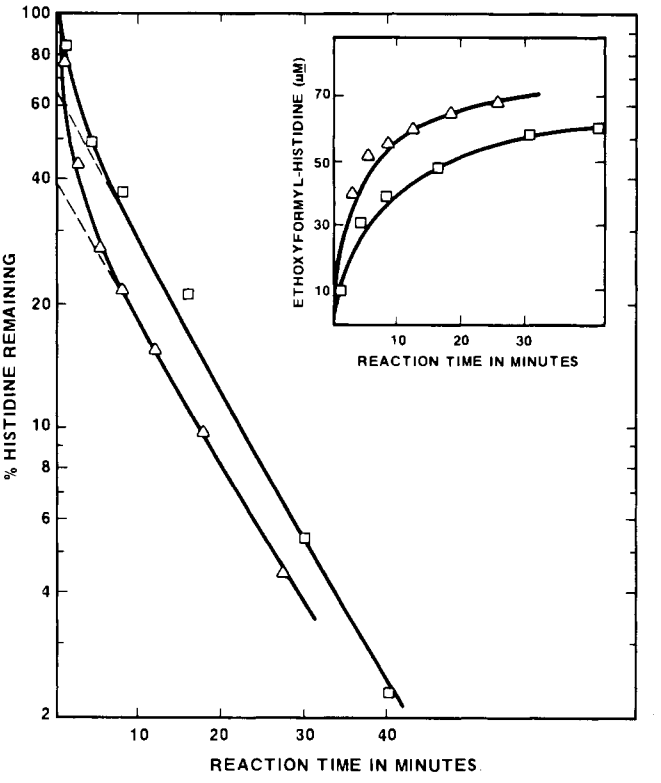

FIGURE 2: Semilogarithmic plot of the loss of histidines in transferrins: human serum apotransferrin (3.90 μM) in the presence of 2.17 mM ethoxyformic anhydride (△); apoovotransferrin (3.90 μM) with 18 mM ethoxyformic anhydride (□). The curves for the slow phase of the reactions are extrapolated to zero time (- - -). Insert: The rates for the formation of ethoxyformylhistidines in the two proteins are shown using the same data as Figure 2. (△) Human serum apotransferrin, (□) apoovotransferrin; ethoxyformylhistidine values normalized such that initial concentrations of histidines are the same for both protein reactions.

same conditions (Figure 1). Thus, the binding of each iron protected two histidines from modification.

Reversal of Ethoxyformylation. Melchior and Fahrney (1970) reported that histidine can be regenerated from ethoxyformylhistidine by incubation with NH_2OH. Modified apoovotransferrin samples were exposed to NH_2OH in order to regenerate histidines and iron-binding activity. The results are shown in Table I. When only 24% of the iron-binding activity was lost after 7 min of modification, most all (94%) of the activity was regained on further incubation with NH_2OH. However, when 56% of the iron-binding activity was lost after 20 min, only part (67%) was regained on further incubation with NH_2OH. Controls were included here to show that NH_2OH treatment conditions did not cause a loss in activity. Tyrosine modification contributing to activity loss was excluded, since spectra taken of ethoxyformylated proteins before and after NH_2OH treatment showed no significant change in absorbance at 278 nm.

Ethoxyformylation of Human Serum Transferrin. The histidines of human serum transferrin were generally more reactive toward ethoxyformic anhydride. Only a 170-fold molar excess of ethoxyformic anhydride was needed to modify a maximum number of histidines. For the apoprotein, 19.5 residues were modified per mole of transferrin, a value 15% higher than the previously reported 17 histidines (Sutton and Brew, 1974). At the same reagent concentration, only 15.6 histidines could be modified in diferric transferrin. As in the case with ovotransferrin, the binding of each iron protected two histidines from modification.

Comparison of Human Serum Transferrin and Ovotransferrin Reactivities Toward Ethoxyformic Anhydride. Parallel experiments were performed with 3.90 μM ovotransferrin or human serum transferrin with 400- and 170-fold molar excesses of reagent to histidines present, respectively. These conditions were chosen so that all histidine residues could be modified with the minimum reagent necessary. The semilog rate curve for the disappearance of histidine is shown in Figure 2. Both reactions are biphasic in nature, with a fast phase nearly complete in the first 3 to 4 min followed by a slow reaction. Analysis of such biphasic reactions as two simultaneous first-order reactions has previously been reported (Ray and Koshland, 1962). The separate rate constants for a fast-reacting or exposed group of histidines and a slower-reacting group of "buried" histidines are calculated by this method from data in Figure 2. The k_{obsd} for the slow phase is very close for both proteins; 0.11 and 0.12 min^{-1} for human serum apotransferrin and apoovotransferrin, respectively. The rate constants for the fast phase could not be calculated accurately, but were >0.90 min^{-1} in both cases. The rate of the slow phase does not depend on the concentration of ethoxyformic anhydride from 3 to 18 mM, so a comparison of the k_{obsd} values can be made despite different reagent concentrations. The fraction of histidines reacting in the slow phase was determined by the intercept of the extrapolated slow reaction portion of the curve (Figure 2). There are ten histidines, or 60%, in human serum

TABLE I: Iron-Binding Activity of Ethoxyformylovotransferrin and Modified Protein Treated with Hydroxylamine.

Sample	Reaction Time (min)	Act. (%)
Ovotransferrin/NH_2OH	0	100[a]
Ethoxyformylovotransferrin	7	76
Ethoxyformylovotransferrin/NH_2OH	7	94[a]
Ethoxyformylovotransferrin	20	44
Ethoxyformylovotransferrin/NH_2OH	20	67[a]

[a] Samples incubated in 0.10 M NH_2OH prior to activity determination.

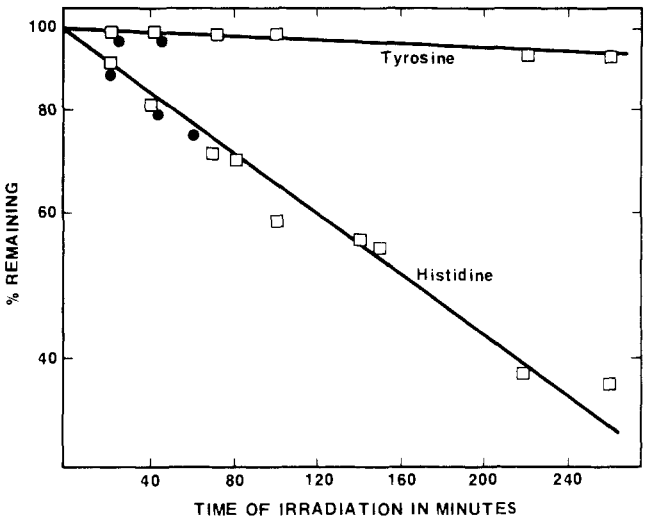

FIGURE 3: Semilogarithmic plot of the losses in histidine and tyrosine upon photooxidation of ovotransferrin. The reaction conditions are as described in the text. Losses in histidine and tyrosine determined by amino acid analysis: (□) apoovotransferrin, (●) diferric ovotransferrin.

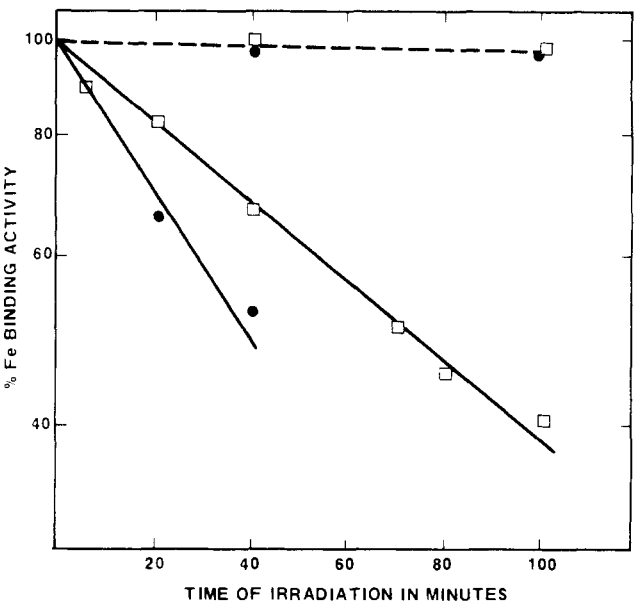

FIGURE 4: Semilogarithmic plot of the losses in iron-binding activity upon photooxidation of ovotransferrins. Experimental conditions are as described in the text. Activity determinations were made by a spectrophotometric titration. Apoovotransferrin (□—□), diferric ovotransferrin (●—●), apoovotransferrin with 10 mM NaN_3 added to reaction mixture (□- - -□), diferric ovotransferrin with 10 mM NaN_3 added (●- - -●).

transferrin involved in the fast phase, while only 4.3 histidines, or 33%, are "exposed" in ovotransferrin.

Losses in Amino Acid Residues upon Photooxidation. When apoovotransferrin was irradiated in the presence of methylene blue, a first-order loss in the amount of histidine in the protein was observed. It was also determined that the rate of loss of tyrosine was very low or essentially zero. These results are shown as semilog rate plots in Figure 3. After 240 min, 35% of the total histidines remained, while the tyrosine level remained at approximately 94% of the original zero-time value. When diferric ovotransferrin was subjected to the same oxidative conditions, the rate of loss of histidine and of tyrosine for the first 60 min was essentially the same as for apoovotransferrin (Figure 3).

Activity Losses upon Photooxidation. A spectrophotometric titration at 470 nm was utilized to determine the iron-binding activity. Activities of the photooxidized ovotransferrin samples are shown in Figure 4. There is a first-order loss in activity upon irradiation of apoovotransferrin. After 100 min, approximately 40% of the iron-binding activity remained. The first-order rate constants for the loss in amino acid residues and activity for apoovotransferrin were calculated from the slope of the semilog plots in Figures 3 and 4. The k_{obsd} values for the destruction of histidine and the loss in activity are 0.0042 and 0.0094 min^{-1}, respectively. The ratio of the rate of loss of histidine to the rate of loss in activity is 2.2. Analysis of this data as previously reported suggests there are two histidines destroyed per binding site which are responsible for the inactivation of ovotransferrin (Ray and Koshland, 1962).

The effect of pH on the photoinactivation of ovotransferrin was also investigated in order to further associate histidines with the inactivation process. Histidine residues' lability to photooxidation is characteristically pH dependent (Spikes and MacKnight, 1970). The photoinactivation of apoovotransferrin showed a profound pH dependence. Identical samples were irradiated for 100 min at various pH values and the following activities were observed: pH 5.5, 82%; pH 6.0, 77%; pH 6.5, 60%; pH 7.35, 45%. This pH profile of inactivation indicates that the destruction of histidine is responsible for the loss in activity.

In order to determine if iron binding protected the protein from inactivation, diferric ovotransferrin was irradiated for various times, and the activity was determined. Not only did the iron fail to protect the protein from photoinactivation, the rate was clearly enhanced (relative to the apoovotransferrin reaction) as shown in Figure 4. The rate of loss of histidine is very similar both for the apoovotransferrin and diferric ovotransferrin samples (Figure 3).

TABLE II: Effect of Metal Binding on Photoinactivation.

Sample	% Act. after 180 min of Irradiation	
	Methylene Blue	No Dye
Apo-OT[a]	82	97
Fe_2-OT	69	93
$Ga(III)_2$-OT	44	93

[a] OT, ovotransferrin.

Effect of Metal Binding on Photoinactivation. The effect of metal binding on the photooxidation mechanism was investigated further, since the data indicated that the holoprotein reacted via a different process. In the presence of 10 mM NaN_3, both diferric ovotransferrin and apoovotransferrin are protected from inactivation as shown in Figure 4, indicating that singlet oxygen is involved as an intermediate in both reactions (Nilsson and Kearns, 1973). Controls were done in which diferric ovotransferrin was irradiated in the absence of dye in order to see if the enhanced inactivation process was associated with the electronic transitions responsible for the color of the iron–protein complex itself. As shown in Table II, these samples were found to be nearly completely active after 180 min of irradiation. Further, in order to exclude the possibility that diferric ovotransferrin inactivation was due to some special effect of the paramagnetic iron in the binding site, colorless, diamagnetic digallium(III)–ovotransferrin complexes were irradiated under the same conditions. The gallium complexes were also less active than the apoovotransferrin samples irradiated under the same conditions (Table II).

Use of Other Dyes. The use of other dyes as photosensitizers was also investigated. Irradiation of apoovotransferrin in the presence of proflavin yielded no significant loss of histidine or tyrosine. The dye tended to associate with the protein, making spectral activity determinations impossible. Rose bengal was not used, since it was immediately bound tightly to the protein and could not be dissociated by either gel filtration or dialysis with various pH and ionic-strength conditions.

Discussion

When ovotransferrin is subjected to either ethoxyformylation or dye-sensitized photooxidation, a gradual loss in histidine and iron-binding activity is observed. The reaction of histidines upon photooxidation proceeded in a random fashion; that is, there did not appear to be any unique reactivity attributable to any of the essential histidines. The reaction of histidines in transferrins with ethoxyformic anhydride showed a biphasic character, a rapid first-order loss of "exposed" groups followed by a slower reacting group of histidines. Contrasting reactivity was also seen toward the two reagents upon the binding of iron. The binding of Fe^{3+} protected some of the histidines from reaction with ethoxyformic anhydride and, correspondingly, the binding activity was unchanged. When ovotransferrin was irradiated in the presence of methylene blue, iron binding had no effect on the rate of loss of histidine. In this case, iron in the binding site showed no protective effect upon photooxidation. In fact, the rate of loss of activity was increased significantly.

All of the histidines in apoovotransferrin and human serum apotransferrin reacted with ethoxyformic anhydride. It was previously reported that all of the histidines in human serum transferrin could be modified only after reduction and carboxymethylation (Krysteva et al., 1975). However, in our experiments much higher molar excesses of reagent were employed (170-fold vs. 15-fold molar excess). In order to demonstrate that the loss in activity could be correlated with a loss in histidine, reversal of the ethoxyformylation of histidines in ovotransferrin was accomplished by incubation with hydroxylamine. These regeneration experiments showed that the removal of the ethoxyformyl group from histidine restored some of the iron-binding activity. An inability to regenerate all of the activity may be due in part to side reactions of ethoxyformic anhydride. In model compounds as well as in proteins, amino groups can react with ethoxyformic anhydride above pH 7 (Larrouquère, 1964; Mühlrad et al., 1967; Rosén et al., 1970).

A recent report shows that cleavage of the imidazole ring of ethoxyformylhistidine is possible in model systems and that the products have a higher absorbance than ethoxyformylhistidines (Loosemore and Pratt, 1976). This reaction may account for the irreversible inactivation by ethoxyformic anhydride with higher levels of reagent as well as the high values for histidine obtained in the human serum transferrin reaction.

In comparing the relative reactivities of ovotransferrin and human serum transferrin toward ethoxyformic anhydride, it is evident that all of the histidines in human serum transferrin could be modified at lower concentrations of reagent. The curves in Figure 2 indicate that the observed rate constants for the fast and slow phases for the modification are very similar for the two proteins. This comparison is possible, despite the fact that different concentrations of ethoxyformic anhydride were used, since the rate for the inaccessible histidines was independent of the reagent concentration in the case of ovotransferrin. This suggests that an event such as protein unfolding is rate limiting in the slow phase. The greater reactivity of human serum transferrin with ethoxyformic anhydride is due in part to the fact that it has a greater percentage of its histidines "exposed" rather than a result of a group of unusually reactive histidines having a large observed rate constant.

Dye-sensitized photooxidation of proteins can result in the destruction of methionine, tryptophan, histidine, tyrosine, and cysteine when methylene blue is used as a sensitizer (Ray, 1967). Upon irradiation of ovotransferrin, only losses in histidine were observed, while tyrosine residues were unaffected by the reaction. Since tyrosines are involved in iron chelation to the protein, the possibility of this side reaction occurring had to be excluded before interpretations on the importance of histidine could be made. On acid hydrolysis, only histidine levels showed a decrease. Hydrolysis (Neumann, 1967; Simpson et al., 1976) and spectrophotometric procedures (Horton and Koshland, 1972) failed to give quantitative yields of methionine and tryptophan, so the fates of these residues are not known. However, tryptophans are reported to be nonessential for iron binding (Ford-Hutchinson and Perkins, 1972). Methionines have never been associated with the iron-binding site (Feeney and Komatsu, 1966; Bezkorovainy and Zschocke, 1974). The pH profile for the photoinactivation is similar to a pH titration curve for histidine. This not only implicates the destruction of histidines as the event responsible for the loss in activity but provides an indirect method for determining the pK_a of essential histidines. An observed inflection point at pH 6.2 to 6.4 is well within the normal pK_a range for histidines in proteins. While complete residue analysis was not possible with respect to tryptophan and methionine, the evidence indicates histidines are essential for the binding of iron.

An interesting result of the photooxidation work was the inability of iron to protect the protein from inactivation. We had expected protection for two reasons. Iron ought to sterically block residues in the active center from exposure to the reagent, and the paramagnetic high-spin Fe^{3+} should quench singlet oxygen (Jori et al., 1969). It is reported that the chelation of zinc and copper protects histidine from photooxidation when bound to protein active centers (Tait and Vallee, 1966; Forman et al., 1973). Since the rate of photoinactivation is clearly enhanced over that for apoovotransferrin, a number of experiments were done in order to define a different photoinactivation mechanism for diferric ovotransferrin. Specific electronic transitions of the iron–protein colored complex were not involved in the photoinactivation. Diferric ovotransferrin samples in the absence of dye showed no activity loss. Also, colorless, diamagnetic Ga(III)–ovotransferrin complexes showed a similar enhanced photoinactivation rate. Thus, this increase in the photoinactivation rate seems to be independent of the type of metal in the binding site, whether paramagnetic or diamagnetic, in a colored or colorless protein complex. The inactivation process for both apoovotransferrin and diferric ovotransferrin involves singlet oxygen, since NaN_3 protects both forms of the protein, excluding the notion of a specific dye–metal interaction in the holoprotein.

It is not clear what difference in the photoinactivation process accounts for the increased reactivity of diferric ovotransferrin compared to apoovotransferrin. The possibility exists that the binding of a metal specifically activates an amino acid residue toward attack by singlet oxygen. For example, tyrosines ionize to the phenolate form, the photolabile species of this amino acid, upon the binding of iron (Warner and Weber, 1953; Weil, 1965). Studies on the effect of metal

chelation upon the rate of photooxidation of amino acids may shed light on this question.

A primary objective of this work was the quantitation of the number of essential histidines at the active site of ovotransferrin. The binding of each iron protected two histidines from ethoxyformylation. This represents a maximum number of essential histidines, since the binding of iron may cause a conformational change, thereby protecting histidines some distance from the active center. The analysis of the kinetic data of the photooxidation reaction indicates that two histidines are essential. This was determined by the observation that the rate constant of inactivation reaction was 2.2 times the first-order rate constant for the destruction of histidine. This value of two essential histidines obtained from kinetic data represents a minimum number of essential residues. There may be other residues of the same type which are nonreactive or very slowly reacting which will not contribute to the kinetic data for inactivation. Thus, by utilizing two different chemical-modification techniques and two distinct methods of analysis of the reaction data, it is shown that two essential histidines are found in each of the iron-binding sites of ovotransferrin.

The functional roles of histidines in proteins have been investigated with varying success utilizing chemical-modification techniques (Means and Feeney, 1971; Hirs and Timasheff, 1972; Glazer et al., 1975). The main problem appears to be the nonspecificity of many reagents making unequivocal interpretations difficult. 5-Diazonium-1H-tetrazole was found to react nonspecifically with transferrin (Line et al., 1967), as well as with other proteins, modifying all 54 of the ϵ-amino groups of lysine and only 60% of the imidazole side chains, the group of interest in this study. Other side reactions with this reagent include extensive modification of tyrosine residues as shown with myoglobin (Takenaka et al., 1970) and RNA polymerase (Zaheer and Nicholson, 1971). This reagent, therefore, should probably not be used to investigate histidines when tyrosines are also essential, as in the case with transferrin. Haloacetates have also been employed in the study of histidines. Bromoacetate readily alkylates ϵ-amino groups and methionines as well as histidines. Even when specificity is obtained, the rates of modification are extremely slow. It required 12 days to alkylate 68% of the histidines in human serum transferrin (Line et al., 1967).

Ethoxyformic anhydride and sensitizing dyes appear to be useful reagents for studying histidines in proteins. Ethoxyformylation is particularly useful as a probe for "exposed", "buried", and substrate-"protected" residues. However, the possibility of imidazole ring cleavage during ethoxyformylation in many protein systems, particularly at high levels of reagent, may prove to be an important consideration (Loosemore and Pratt, 1976). When a large number of histidines are involved, as is the case with large proteins like transferrin, photooxidation provides a method for kinetic analysis of the reaction by adjustment of the reaction conditions. Both of these techniques used in concert provide an approach to the study of the chemical nature of histidines that should yield fruitful information. In general, our studies further support the notion that all studies using chemical modification of a specific type of residue should include methods fundamentally different from one another.

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Binding Specificity of the Juvenile Hormone Carrier Protein from the Hemolymph of the Tobacco Hornworm *Manduca sexta* Johannson (Lepidoptera: Sphingidae)†

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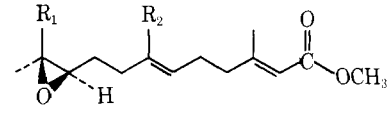
ABSTRACT: A series of analogues of insect juvenile hormone (four geometric isomers of methyl epoxyfarnesenate, several para-substituted epoxygeranyl phenyl ethers, and epoxyfarnesol and its acetate and haloacetate derivatives) was prepared to investigate the binding specificity of the hemolymph juvenile hormone binding protein from the tobacco hornworm *Manduca sexta.* The relative binding affinities were determined by a competition assay against radiolabeled methyl (*E,E*)-3,11-dimethyl-7-ethyl-*cis*-10,11-epoxytrideca-2,6-dienoate (JH I). The ratio of dissociation constants was estimated by plotting competitor data according to a linear transformation of the dissociation equations describing competition of two ligands for a binding protein. The importance of the geometry of the sesquiterpene hydrocarbon chain is indicated by the fact that the binding affinity is decreased as *Z* (cis) double bonds are substituted for *E* (trans) double bonds in the methyl epoxyfarnesenate series; the unepoxidized analogues do not bind. A carboxylic ester function is important although its orientation can be reversed, as indicated by the good binding of epoxyfarnesyl acetate. In the monoterpene series, methyl epoxygeranoate shows no affinity for the binding protein, but substitution of a phenyl or *p*-carbomethoxyphenyl ether for the ester function imparts a low, but significant affinity. These data taken together with earlier results indicate that the binding site for juvenile hormone in the hemolymph binding protein is characterized by a sterically defined hydrophobic region with polar sites that recognize the epoxide and the ester functions.

Insects undergo extensive postembryonic development and thus provide intriguing experimental systems for exploring control of developmental events. The regulation of these processes by two insect hormones, juvenile hormone (JH[1]) and ecdysone, the molting hormone, has been the focus of much attention.

The structures of the three known insect juvenile hormones are shown below. Juvenile hormone acts during larval stages



JHI, $R_1 = CH_2CH_3$; $R_2 = CH_2CH_3$ (Röller et al., 1967)
JHII, $R_1 = CH_2CH_3$; $R_2 = CH_3$ (Meyer et al., 1968)
JHIII, $R_1 = CH_3$; $R_2 = CH_3$ (Judy et al., 1973)

by preventing the growth and differentiation of those cells destined to form typical adult structures. Hormone titer remains high during early larval stages but decreases dramatically in the last larval stage. The absence of JH at this time allows expression of genetic information leading to metamorphosis from the larva to the pupa.

In the hemolymph of the tobacco hornworm, *Manduca sexta,* a specific binding protein carries JH from the site of synthesis, the corpus allatum, to target tissues throughout the

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[1] Abbreviations used are: JH, juvenile hormone; JH I, methyl (*E,E*)-3,11-dimethyl-7-ethyl-*cis*-10,11-epoxytrideca-2,6-dienoate; JH II, methyl (*E,E*)-3,7,11-trimethyl-*cis*-10,11-epoxytrideca-2,6-dienoate; JH III, methyl (*E,E*)-10,11-epoxyfarnesenate; DEAE, diethylaminoethyl; THF, tetrahydrofuran; NMR, nuclear magnetic resonance; IR, infrared; GLPC, gas–liquid partition chromatography; TLC, thin-layer chromatography; PLC, preparative thin-layer chromatography; Tris, tris(hydroxymethyl)-aminomethane.