RESISTANCE OF METAL COMPLEXES OF CONALBUMIN AND TRANSFERRIN TO PROTEOLYSIS AND TO THERMAL DENATURATION*

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The iron-binding proteins, conalbumin of egg white and transferrin of plasma, form very similar, red-colored, stable complexes with 2 atoms of ferric iron (1–10). The formation of the iron complex requires 1 molecule of CO₂ as CO₂⁻ or HCO₃⁻ per atom of Fe³⁺, and the second iron atom is bound more strongly than the first (6). Although the reactive groups have not been identified, the hydroxyl groups of tyrosine have been implicated (6, 9). All types of chemical reagents and denaturing conditions were found to reduce the affinity of conalbumin for iron, and it was concluded that the particular spatial configuration of the native protein was required for the formation of the colored metal complexes (4). The two proteins also form relatively weaker complexes with Cu²⁺ and Zn²⁺. Complex formation might be expected to stabilize the structure of a protein in some cases, and two isolated observations have been made as to the relatively greater stability of iron conalbumin as compared to free conalbumin. Fraenkel-Conrat (10) indicated a greater stability to trypsin, and Warner and Weber (6) reported a greater stability under alkaline conditions.

In the present study, a much greater stability of the metal complexes of both conalbumin and transferrin was encountered than had apparently been suspected heretofore. This report concerns the relative stabilities of these proteins and their metal complexes to enzyme action and to thermal denaturation.

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1 Transferrin is also known by the following names: siderophilin, β₁-metal-combining (pseudo) globulin, or simply as the iron-combining component of plasma.
Proteins and Enzymes—Conalbumin was prepared in crystalline form from egg white by the batch method of adsorption on carboxymethylcellulose as recently described (11). The nitrogen content was 16.4 per cent in terms of dry weight, and the lysozyme content was <0.001 per cent (11, 12). All preparations were homogeneous by free boundary electrophoresis.

The transferrin was obtained in a highly purified state, but not crystalline, from the Cutter Laboratories. It had been prepared by alcohol fractionation (7, 9), and its chromogenic capacity with iron was approximately three-fourths that of crystalline conalbumin. Lysozyme was crystallized from egg white according to Alderton and Fevold (13). Crystalline bovine serum albumin was obtained from the Armour Laboratories. The twice crystallized chymotrypsin and trypsin were obtained from the Worthington Biochemical Corporation.

Solution of Metal Ions—The concentrated stock solutions of metal ions (0.02 or 0.05 mM Fe++, Zn++, Cu++) were prepared, respectively, from ferric nitrate, zinc acetate, and copper acetate dissolved in a mixture of 0.1 M sodium citrate and 0.1 M bicarbonate.

Spectrophotometry, Paper Chromatography, and Electrophoresis—A Beckman model DU spectrophotometer equipped with a photomultiplier attachment was used for all spectrophotometric determinations. Trypsin activity was determined spectrophotometrically with p-toluenesulfonylarginine methyl ester as the substrate according to the rapid method of this laboratory (14).

Chromatograms were obtained by the descending method on Whatman No. 1 paper with a butanol-acetic acid-water (4:1:5) mixture (15). The papers were sprayed with 0.1 per cent ninhydrin in ethyl alcohol containing 5 per cent collidine, and the color was developed by brief heating over a hot plate. Moving boundary and paper electrophoretic studies were performed essentially as described elsewhere (11).

Enzymatic Hydrolytic Experiments—Enzymatic hydrolyses of conalbumin

2 After this study was under way, it came to our attention that the Cutter Laboratories remove impurities from transferrin for immunological purposes by heating the iron complex.

3 The concentrations and amounts of proteins and metal ions are given in weights for convenience in comparing proteolytic effects. On a weight basis, 1.0 mg. of conalbumin binds approximately 1.35 \( \gamma \) of Fe++. This forms the saturated complex with 2 atoms of iron per molecule of conalbumin and is described in this communication as iron conalbumin or Fe conalbumin. The saturated complex of transferrin is also referred to in this manner. The incompletely saturated mixture with 1 atom of iron per molecule of protein is indicated by the numbers in parentheses as follows: iron (1:1) conalbumin. All buffers used were as the sodium salts, unless otherwise indicated.
and the metal complexes of conalbumin were performed in a buffer of 0.1 M tris(hydroxymethyl)aminomethane at pH 8.0 or in a mixed buffer of 0.05 M phosphate and 0.05 M carbonate at pH 8.0 to 9.0. Concentrations of conalbumin, metal ions, and other added substances in the solutions employed were adjusted to give the desired final concentrations. In the individual experiments, water or metal ions were added to the buffered solutions of conalbumin, and the mixed solutions were incubated in a thermostatically controlled bath for 15 to 30 minutes. The optical density was then determined at 470 nm for iron conalbumin or at 440 nm for copper conalbumin. The enzyme was then added to the solutions, and the reaction mixtures were incubated for the desired periods of time. At the completion of the incubation period, the optical density of the solutions containing iron or copper was determined. A 2- to 4-fold excess amount of iron solution was then added to all solutions. The color was allowed to develop for 30 minutes, and the optical densities were determined at 470 nm. Such values were termed color capacities. Color capacity was employed as the criterion for metal binding because of its relative simplicity and because it is closely, if not completely, related to the affinity of the protein for the metal which in turn is a property of the native protein (4).

Thermal Denaturation Experiments—Experiments on thermal denaturation were performed in much the same way as were the enzymatic hydrolytic studies with the principal exception that, instead of adding enzymes to the solutions, the solutions were incubated in a hot water bath. Rapid temperature equilibration was aided by adding concentrated solutions of the proteins to tubes which contained 10 to 20 volumes of the buffer heated to the desired temperature.

Results

Resistance of Conalbumin to Chymotrypsin and to Trypsin—The color capacity of metal-free conalbumin was rapidly destroyed by incubation with either chymotrypsin or trypsin, but the color capacity of iron conalbumin was unaffected under the same treatments even after prolonged times of incubation. The results of a typical experiment with chymotrypsin are given in Fig. 1.

The losses of color capacity of the metal-free conalbumin with enzyme treatment were accompanied by hydrolytic fragmentation to peptides. Peptide formation was demonstrated by paper chromatography, and the amount and degree of fragmentation (as estimated by the number of spots and their intensity) were roughly proportional to the amount of loss of color capacity.

The enzyme-treated iron conalbumin (and the metal-free conalbumin prepared therefrom), however, was found identical to original untreated
material by the following criteria: capacity for being crystallized and crystallinity, moving boundary and filter paper electrophoretic analysis, paper chromatography and column chromatography on carboxymethyl-cellulose. The enzyme-treated material from which both the enzyme and iron had been subsequently removed was also examined for rate of color formation with iron and for rate of hydrolysis with chymotrypsin and was found similar to the untreated conalbumin.

![Diagram](http://www.jbc.org/)

**FIG. 1.** Loss of chromogenic capacity of iron conalbumin and conalbumin in the presence of chymotrypsin. The reaction was carried out in 0.1 M tris(hydroxy-methyl)aminomethane buffer, pH 8.0, containing 4 mg. of conalbumin per ml. The iron conalbumin solution contained 8 μg of Fe*++* per ml. The solutions were incubated at 37° at pH 8.1 for 12 hours. The residual color capacity of conalbumin was determined after the addition of excess iron to an aliquot of the incubated mixture at any given time. The enzyme-conalbumin weight ratios were as represented on the curves.

Other metal-ion complexes of conalbumin were also resistant. Their resistances, however, were considerably less than that of iron conalbumin and were inversely proportional to the strengths of the complexes. Thus, the weak copper complex was hydrolyzed at a rate approximately 1 per cent that of metal-free conalbumin, and the still weaker zinc conalbumin was hydrolyzed at a rate approximately 20 per cent that of metal-free conalbumin. Iron (1:1) conalbumin was rapidly attacked until 50 per cent hydrolysis occurred.4

4 This is in agreement with the existence of only the saturated iron complex (2 atoms of iron per molecule of conalbumin). Iron (1:1) conalbumin would therefore be a mixture of 50 per cent conalbumin and 50 per cent iron conalbumin. Another possibility might be that the mixture initially exists as 100 per cent iron (1:1) con-
In comparative studies with trypsin, iron conalbumin was similarly found resistant to hydrolysis (Fig. 2).

Proof for Non-Inhibition or Non-Inactivation of Enzymes by Iron or Iron Conalbumin—Proof for the non-inhibition of the enzymes by iron conalbumin or excess iron was based on the following: (a) Bovine serum albumin or lysozyme was attacked by chymotrypsin in the presence of iron conalbumin or ferric iron. The rates of hydrolysis and hydrolytic patterns (as measured by paper chromatography) were approximately the same in the presence or in the absence of iron conalbumin. (b) The addition of iron conalbumin did not affect the rate of hydrolysis of p-toluene-sulfonylarginine methyl ester by trypsin. (c) Any amounts of conalbumin in excess of the amount required to form the saturated iron complex in different mixtures were hydrolyzed by chymotrypsin.

albumin which has approximately the same adsorption spectrum and extinction coefficient at 470 m\(\mu\) as the saturated complex has and which is not resistant to proteolysis. As each molecule of the labile 1:1 complex would be hydrolyzed, the iron could then be bound by another 1:1 complex to form the stable saturated complex. The theoretical mixture obtained after “maximal” hydrolysis would therefore be 50 per cent saturated complex and 50 per cent hydrolyzed products. No evidence for the 1:1 complex, however, has been observed in this study.
Proof for non-inactivation of the enzymes was based on the following: (a) When additional conalbumin (metal-free) was added to incubated solutions of iron conalbumin and chymotrypsin (which had shown no evidence of hydrolytic action), hydrolysis quickly occurred. (b) When a 20-fold excess of ethylenediaminetetraacetic acid was added to incubated solutions of iron conalbumin and chymotrypsin, slow hydrolysis occurred, in agreement with the slow removal of iron from iron conalbumin under such conditions. (c) Iron conalbumin did not increase the rate of loss of trypsin activity (as measured with p-toluenesulfonylarginine methyl ester) occurring on incubation under the conditions employed for the proteolytic studies.

**Resistance of Iron Conalbumin to Thermal Denaturation**—Iron conalbumin was found much more resistant to thermal denaturation in solution than metal-free conalbumin. From comparisons of the data of the experiment given in Table I and other similar experiments, an approximate estimation of the relative resistance at 64° was that metal-free conalbumin was denatured at least a thousand times faster than iron conalbumin. This, of course, is a minimal figure.

Limited studies on the stabilities of copper and zinc conalbumin to

### Table I

**Resistance of Iron Conalbumin to Heat**

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Material tested</th>
<th>Conditions</th>
<th>Results†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Temperature</td>
<td>Time</td>
</tr>
<tr>
<td>1</td>
<td>Fe conalbumin</td>
<td>64°</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>&quot;</td>
<td>64°</td>
<td>30</td>
</tr>
<tr>
<td>3</td>
<td>&quot;</td>
<td>64°</td>
<td>60</td>
</tr>
<tr>
<td>4</td>
<td>&quot;</td>
<td>64°</td>
<td>120</td>
</tr>
<tr>
<td>5</td>
<td>&quot;</td>
<td>75°</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>Conalbumin</td>
<td>60°</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>&quot;</td>
<td>63°</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>&quot;</td>
<td>64°</td>
<td>2</td>
</tr>
</tbody>
</table>

*Performed in a mixed buffer of carbonate (0.1 M), citrate (0.9 mM), and phosphate (0.075 M) at pH 8.4. The conalbumin concentration was 6.25 mg. per ml. Fe conalbumin was formed by adding 10 γ of Fe+++ per ml. Excess iron was added to all tubes after incubation and before determination of the optical densities.

† Optical density read after the addition of excess Fe+++. Coagulation = visible coagulation, estimated from 0 to 4+.

‡ This figure is an approximation.

§ These samples probably had an optical density <0.10.
heat showed that their relative resistances were similar to their relative resistances to chymotrypsin. The relative stability of the 1:1 complex of iron with conalbumin also was similar.

The absence of denaturation on heating the iron complex under conditions which caused no loss in color capacity was confirmed by studies of the susceptibility to proteolysis. The heated iron complex was found to be as resistant to proteolysis as the original unheated complex. In one such experiment, iron conalbumin was heated for 30 minutes at 64° under the conditions given in Table I. The sample was cooled, chymo-

**Table II**

*Resistance of Iron Transferrin to Chymotrypsin*

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Incubation mixture*</th>
<th>Optical density after incubation for†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 hr.</td>
</tr>
<tr>
<td>1</td>
<td>Fe transferrin</td>
<td>0.27</td>
</tr>
<tr>
<td>2</td>
<td>&quot; &quot; + chymotrypsin</td>
<td>0.27</td>
</tr>
<tr>
<td>3</td>
<td>Transferrin</td>
<td>0.28</td>
</tr>
<tr>
<td>4</td>
<td>&quot; + chymotrypsin</td>
<td>0.13</td>
</tr>
</tbody>
</table>

* Performed in mixed buffer of carbonate (0.04 M), phosphate (0.02 M), tris(hydroxymethyl)aminomethane (0.03 M), and ethylenediaminetetraacetic acid (1.0 mM) at pH 8.0. The transferrin concentration was 6.0 mg. per ml.; chymotrypsin was 0.3 mg. per ml. Fe transferrin was formed by adding 8 γ of Fe+++ per ml.

† The incubation temperature was 37°. The optical densities of Tubes 1 and 2 were read at the times indicated and again at 6 hours after the addition of excess Fe+++ citrate (22 γ Fe+++ per ml.); no differences in optical density were found. The optical densities of Tubes 3 and 4 were read by the addition of excess Fe+++ citrate at the times indicated.

trypsin was added to it, and the mixture was incubated at 37°. No hydrolysis occurred during periods of incubation in which extensive hydrolysis of controls of metal-free conalbumin occurred. In further experiments it was found that, when iron conalbumin was heated at high temperatures for a sufficient time to cause partial losses of color capacity, treatment of the reaction mixtures with chymotrypsin caused no further decreases in color capacity. Hydrolysis occurred, however, and the amounts of hydrolysis (as measured by the amounts of trichloroacetic acid-soluble substances) were proportional to the amounts of loss in color capacity caused by heating.

*Resistance of Iron Transferrin to Chymotrypsin*—The resistance of iron transferrin and metal-free transferrin to chymotrypsin was compared in experiments similar to those described above for conalbumin. The iron
transferrin was also much more resistant to chymotrypsin than the metal-free protein (Table II). There was usually, however, a slight decrease in the color capacity of iron transferrin.

Resistance of Iron Transferrin to Thermal Denaturation—Iron transferrin was much more resistant to thermal denaturation than the metal-free protein, a relationship also similar to that for iron conalbumin and conalbumin. Table III presents the data of one of several experiments on this subject. There was no apparent fundamental difference in the comparative resistances of iron transferrin and iron conalbumin to heat, but

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Tube No.</th>
<th>Material tested</th>
<th>Conditions</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Temperature</td>
<td>Time</td>
</tr>
<tr>
<td>A</td>
<td>1</td>
<td>Fe transferrin</td>
<td>°C. 0 min.</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>&quot; &quot; &quot;</td>
<td>65 120 min.</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Transferrin</td>
<td>65 10 min.</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>&quot; &quot; &quot;</td>
<td>65 30 min.</td>
<td>0.13</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>Fe transferrin</td>
<td>°C. 0 min.</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>&quot; &quot; &quot;</td>
<td>67.3 30 min.</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>&quot; &quot; &quot;</td>
<td>67.3 60 min.</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Transferrin</td>
<td>67.3 2 min.</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>&quot; &quot; &quot;</td>
<td>67.3 5 min.</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>&quot; &quot; &quot;</td>
<td>67.3 10 min.</td>
<td>0.14</td>
</tr>
</tbody>
</table>

Performed under the conditions for the data of Table I. The transferrin concentration was 6.25 mg. per ml. (uncorrected basis).

the metal-free proteins differed in respect to the conditions causing coagulation on heating.

DISCUSSION

Metal ions have been reported to increase and to decrease the stabilities of different proteins, and various causes have been attributed to these effects (16-20). It is possible that the resistances of the metal complexes of conalbumin and transferrin to both proteolysis and thermal denaturation are due to different causes, such as the blocking of sites susceptible to enzymic cleavage and the stabilization of a physical structure to thermally induced derangements. It is also possible, however, that the resistances to the two treatments are caused by the same fundamental change, such as one in molecular shape or charge distribution. In this connection,
Fuller and Briggs reported that comparisons of the frictional ratios of conalbumin and iron conalbumin indicated that iron conalbumin was the more nearly spherical (8). There are, of course, many factors influencing the stability of proteins (21).

The recent theories of Linderstrøm-Lang (22) on the relationship between proteolysis and protein denaturation support the second possibility discussed above, i.e. a similar reason for the resistance to both treatments. Gurd and Wilcox (16) modified the theoretical scheme of Gorini and Audrain (19) for resistance to proteolysis to include resistance to denaturation as well as to proteolysis. A still further modification to fit the results of the present study is the following:

\[
A + M \rightleftharpoons AM
\]

\[
\uparrow
\]

\[
B (B_1, B_2, B_3, \text{etc.})
\]

heat / proteolysis

\[
D \quad P
\]

In the above equation, \(A\) is the native protein which is resistant\(^6\) and \(B\) is the configuration which is susceptible to proteolysis to give products, \(P\), or to denaturation to give the denatured form, \(D\). The metal ion, \(M\), combines with \(A\) to form \(AM\), which is so slightly dissociated that \(B\) is practically non-existent (in the case of the iron complexes). The greater resistances of the metal complexes would thus both be due to a greater resistance to an initial physical change, i.e. to the formation of \(B\) (or \(B_1, B_2, \text{etc.}\)).

**SUMMARY**

1. The iron complexes of the egg white protein, conalbumin, and the \(\beta_1\)-metal-binding protein from human serum, transferrin, were both found much more resistant to denaturation by heat and to hydrolysis by chymotrypsin or trypsin than were the metal-free proteins. The resistances of the copper and zinc complexes were in proportion to the stabilities of the complexes.

2. The results were considered as possible evidence that the resistances to proteolysis and heat denaturation were both caused by the same fundamental change in the properties of the protein occurring on complex

\(^{6}\text{We have modified the scheme as presented by Gurd and Wilcox (16) in several respects. A reaction of } M \text{ with } B \text{ to give } BM (B + M \rightleftharpoons BM) \text{ has been omitted because the data of the present investigation indicated that a complex similar to } BM \text{ either did not exist or was labile to proteolysis. We have also added other possible forms of } B (B_1, B_2, \text{etc.}) \text{ because the data did not preclude their existence.}\)
formation. The possibility that complex formation greatly reduces the amount of a labile intermediate configuration in equilibrium with the normal native configuration was discussed.

The assistance of Nelle Bennett and Rex Bosley in various phases of this investigation and the supervision of the moving boundary electrophoretic determinations by Dr. R. M. Hill are greatly appreciated. Appreciation is also due to the Cutter Laboratories for the supplies of the transferrin.

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