Nonequivalence of the Metal Binding Sites of Conalbumin

CALORIMETRIC AND SPECTROPHOTOMETRIC STUDIES OF ALUMINUM BINDING

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Differential scanning calorimetric experiments show that addition of Al(III) to conalbumin increases its denaturation temperature by 5°, from 63 to 68°. Only one Al(III) bound per conalbumin molecule produces this change in heat stability; additional bound Al(III) does not affect the heat stability. Since Al(III) displaces both Cu(II) bound at the metal binding sites of conalbumin, binding of aluminum takes place at the same metal binding sites. The binding constant for the second Al(III) is at least 100-fold less than that for the binding of the first Al(III), and both are displaced by added iron. The order of increasing heat stability of the metal ion complexes of conalbumin, Cu(II), Al(III), Fe(III), is the order of increasing binding constant for these metal ions.

MATERIALS AND METHODS

Conalbumin was prepared from the white of fresh eggs from White Leghorn chickens by modification of the method of Azari and Haugh (3). DSC thermograms of conalbumin showed no endotherms for denaturation of the other egg white proteins. The ultraviolet absorption of solutions of this preparation agreed with the expected absorption in the visible range when the conalbumin was saturated with iron, indicating absence of other protein contaminants. Absorption coefficients (ε) employed were 11.3 at 280 nm, 11.0 at 280 nm in 0.2 M HCl (4), 11.0 at 280 nm in 0.2 M HCl (4), 0.621 at 470 nm for iron conalbumin at pH 8.5 (4), 0.05 at 440 nm for copper conalbumin at pH 8.5 (5). A molecular weight of 76,600 (6, 7) was assumed for conalbumin.

Chemicals were reagent grade. Distilled water was deionized by passage through a Barnstead mixed-bed ion exchange cartridge. All solutions were air-equilibrated.

Conalbumin solutions were prepared in 0.6 M Tris, pH 7.5, and in 0.1 M Tris, pH 8.5. Concentrations were determined from the ultraviolet absorption of dilutions of aliquots. Solutions of Al(SO₄)₂·16H₂O, Fe(NH₄)₂(SO₄)₂·6H₂O, and CuSO₄·5H₂O, 0.02 to 0.05 M in water, were added in increments to these conalbumin solutions to obtain partial to full saturation with metal ion. Fe(II) in 0.6 M Tris buffer at pH 7.5 is rapidly converted to Fe(III) bound to conalbumin (8, 9).

In a typical calorimetric experiment, small weighed aliquots of Al(III) solution were added in succession to 0.500 ml of conalbumin stock solution. After each addition, samples of 20 μl were weighed into DSC hermetic pans and the pans were sealed. The pH of the conalbumin solution was determined with a Radiometer pH meter, model 26, or a Beckman Research model pH meter, both equipped with Beckman microelectrodes (No. 390600). The Tris buffer was sufficiently concentrated that pH changes did not exceed 0.05 pH unit when metal ions were added. The pH reported is the pH at room temperature.

DSC thermograms were recorded on a DuPont model 990 thermal analyzer. The reference material was a sealed pan containing an equal weight of water. The calibration of the instrument has been described previously (10, 11). As we have observed in studies of other protein solutions, the onset of denaturation is relatively insensitive to changes in programming rate, but the temperature of maximum deflection (maximum heat flow into the sample), referred to here as the "peak temperature", shifts upward with increased heating rate. We report as T_d the peak temperature obtained with the usual programming rate of 10°/min; since the denaturation is irreversible, the peak temperature is a function of heating rate (11).

Replicate samples of conalbumin and conalbumin fully saturated with iron or with aluminum (at least 2-fold excess of metal ion) were used to determine the respective peak temperatures and enthalpies of denaturation. Temperatures were reproducible to better than 1°, and areas of replicate scans, measured with a planimeter, were reproducible within 2%. Enthalpies of denaturation (ΔH_d) were calculated as described previously (10). The most sensitive range of the instrument, 0.05 mcal/s-inch of vertical displacement on the recording chart, was generally employed. The calibration coefficient was determined to vary from 0.96 to 0.98 over the temperature range 63-84°. The estimated error in determining this coefficient is ±2%. Calculation of relative areas under overlapping peaks was made by fitting the observed thermograms with bands having the characteristic shapes of conalbumin and aluminum-conalbumin, at their respective denaturation temperatures.

Spectra and measurements of the kinetics of absorbance changes were determined with a Cary 15 spectrophotometer. Metal ion solu-
tions were manually mixed with conalbumin solutions, so absorption changes which took place in the first 10 s were not observed. A continuous time recording of the absorbancy change at a fixed wavelength was followed by recording of the spectrum in the visible region at completion of the absorbance change. Measurements were made with unthermostatted cells at room temperature (22°).

A kinetic analysis of the spectrophotometric data obtained on displacement of Cu(I) by Al(III) could only be carried out properly after a correction for the change in turbidity of the solution with time was applied. A first order change in turbidity with time was found to be a satisfactory fit to the measurements obtained at longer times. Since the half-times for decrease in turbidity were 2 to 6 × 10^4 s, the assumption of a linear change in turbidity with time would not have significantly affected subsequent calculations. The turbidity was extrapolated back to shorter times, and subtracted from the observed absorbances. The reduced data thus obtained were subjected to standard graphical analysis. The change in absorbance with time was observed to be consistent with two simultaneous first order processes. Best straight lines on semilog plots were fit by inspection.

RESULTS

Fig. 1 shows thermograms for conalbumin and conalbumin partially and fully saturated with aluminum ion. It is apparent that saturation of conalbumin with aluminum increases its heat stability, the peak temperature increasing from 63 to 68°. Enthalpies of denaturation are given in Table I. The heat stabilization of conalbumin by Fe(III) has been reported (12).

To resolve the question of where the Al(III) was bound to the conalbumin molecule, duplicate solutions of conalbumin were prepared. An excess of Al(III) was added to one (in excess of 2 Al(III)/conalbumin). The other was untreated. Addition of excess ferrous ion to the latter solution produced an immediate increase in absorption which was very close to one-half that observed for the apo-conalbumin (Fig. 3). There followed a slow (t₁/₂ ∼500 s) increase in absorption to the same final value of Aₐₐ₉ obtained when ferrous ion was added to apo conalbumin. The subsequent determination of the absorption spectrum revealed that, in both cases, the visible absorption was characteristic of iron-conalbumin.

The experiment just described could equally well be interpreted to mean that either only one Al(III) was bound to conalbumin and was displaced by Fe(III), or that one of two bound Al(III) was displaced extremely rapidly by Fe(III), and the other more slowly. To distinguish between these possibilities, a 2-fold excess of Cu(II) (4 mol of Cu(II) per mol of conalbumin) was added to a conalbumin solution at pH 8.5. Visible and ultraviolet absorption spectra before and after addition of Cu(II) showed that saturation of conalbumin with Cu(II) was essentially complete (97% of calculated Aₐₐ₉). Addition of a 2-fold excess of Al(III) (4 mol of Al(III)/mol of conalbumin) resulted in a complete loss of yellow color observed visually; a recording of the spectrum showed that 5% of the original absorption at 440 nm characteristic of Cu(II)-conalbumin remained (Fig. 4A).

A number of variations on the spectrophotometric observation of the displacement of Cu(II) by Al(III) were carried out in attempts to rule out possibilities such as the displacement of two Cu(II) by one Al(III). Kinetic analysis was complicated by turbidity formation when Al(III) was added to Cu-conalbumin. It appeared that this turbidity was a finely divided precipitate of copper hydroxide. After a time sufficient for the turbidity to settle (usually overnight), the following observations were made with unthermostatted cells at room temperature (22°).

### Table I

<table>
<thead>
<tr>
<th>Species</th>
<th>T°</th>
<th>ΔH° kcal/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conalbumin</td>
<td>63.0</td>
<td>320</td>
</tr>
<tr>
<td>Aluminum-conalbumin</td>
<td>68.0</td>
<td>380</td>
</tr>
<tr>
<td>Diferric-conalbumin</td>
<td>83.0</td>
<td>630</td>
</tr>
<tr>
<td>Conalbumin</td>
<td>64.0</td>
<td>900</td>
</tr>
<tr>
<td>Aluminum-conalbumin</td>
<td>72.5</td>
<td>400</td>
</tr>
<tr>
<td>Dicupric-conalbumin</td>
<td>68.0</td>
<td>330</td>
</tr>
</tbody>
</table>

* At a heating rate of 10°/min.
* Average precision ± 10 kcal/mol.
* In 0.6 M Tris buffer.
* Either one or two Al(III) bound per conalbumin molecule.
* In 1 M Tris buffer. Cupric ion is not bound stoichiometrically to conalbumin at pH 7.5.

Fig. 1. Effect of Al(III) on the heat stability of conalbumin. The thermograms are labeled with the percentage saturation of conalbumin with Al(III), assuming 1 Al(III) per conalbumin molecule at full saturation. Conalbumin concentration, 65 to 75 mg/ml, pH 7.5. Heating rate, 10°/min.

Fig. 2. Stoichiometry of binding of Al(III) to conalbumin. The ordinate gives the relative change from the upper thermogram to the lower thermogram in Fig. 1, measured by the curve-fitting procedure described in the text.
made. One equivalent of Al(III) added either before or after 1 eq of Cu(II) did not prevent near-stoichiometric binding of Cu(II), as measured by $A_{440}$. A 3-fold excess of Cu(II) added subsequently did not increase $A_{440}$ above that calculated for the single Cu-conalbumin complex, assuming an extinction coefficient one-half of that reported for the dicupric complex (5). One equivalent of Al(III) added after 2 eq of Cu(II) caused $A_{440}$ to decrease by one-half. A 2nd eq of Al(III) eliminated most of the remaining $A_{440}$, but displacement of Cu(II) was not complete under these conditions (see above).

A kinetic analysis of the spectrophotometric observation of the displacement of Cu(II) from Cu$_2$-conalbumin by an excess of Al(III) was carried out after correction for change in turbidity with time (see "Materials and Methods"). The decrease in absorbance appears to follow simultaneous first order kinetics. Two experiments carried out on separate stock solutions, 6 weeks apart, gave very similar results (Fig. 4B). First order half-times of the absorbance decrease were 40 and 42 s for the faster process, and 205 and 230 s for the slower process. The absorbance change for each process was the same, within an experimental precision of ±10%.

**DISCUSSION**

The results presented in Fig. 1 are strikingly different from those obtained when iron is added to conalbumin. Four endotherms are observed at partial saturation with Fe(III) (1), but only two are observed at partial saturation with Al(III). The near-identity of $T_d$ for aluminum-conalbumin (68$^\circ$, Table I), with the $T_d$ of 68$^\circ$ observed for one of the single-iron conalbumin species (1) suggests that conalbumin binds only one Al(III). The titration curve of Fig. 2 suggests the same thing. The sharp break in the curve at one Al(III) per conalbumin, followed by a plateau, indicates that only one Al(III) contributes to the increase in heat stability of the conalbumin molecule. This experiment does not reveal, however, whether other Al(III) ions bind less strongly to conalbumin without effect on its heat stability.

Binding of aluminum ion to conalbumin produces no change in absorption in the visible wavelength region. To determine whether the aluminum was bound at one of the iron binding sites of conalbumin, we resorted to displacement of aluminum by iron. The kinetic results (Fig. 3) show that Al(III) prevents half the immediate color development, and so presumably occupies at least one of the iron-binding sites. Since the full color characteristic of iron-conalbumin is slowly attained, the aluminum is slowly displaced from this site by iron. An aluminum ion, if bound at the other iron binding site, is easily displaced by iron and does not contribute to the heat stability. These kinetic experiments, of course, give no information on whether additional aluminum ions are bound at other nonspecific sites on conalbumin.

That two Al(III) are actually bound by conalbumin is revealed by the displacement of the two bound Cu(II) (13, 7) by aluminum (Fig. 4). Thus, one Al(III) is bound at each of the two metal ion binding sites of conalbumin, but only the Al(III) bound more strongly affects the heat stability of the protein.

The absence of curvature near the equivalence point in the plot of Fig. 2 indicates that the binding constant for the second Al(III) must be a factor of 100 or more smaller than the binding constant for the first. The binding constants for the two Fe(III)
ions have been reported to differ by a factor of 12 (14), and those for the two Cu(II) ions by a factor of 20 (7). From the amount of absorption remaining at 440 nm when equal amounts of Al(III) and Cu(II) are present (Fig. 4), an estimate can be made of the ratio of the binding constant for the more weakly bound Al(III) to the more strongly bound of the two Cu(II). This ratio is at least 30, and is probably greater than 100, since equilibrium might not have been attained. Similarly, the binding constant for the more weakly bound of the two Fe(III) must be at least 100 times greater than that of the more strongly bound of the two Al(III). These considerations would place the site binding constants for Al(III) between $10^{21}$ and $10^{22}$, smaller than those for Fe(III) (approximately $10^{29}$, Ref. 7) and larger than those for Cu(II) (approximately $10^{28}$, Ref. 7).

The heat stability of the metal ion complex of conalbumin appears to bear a direct relation to the binding constant for the metal ion, since the order of increasing heat stability, Cu(II), Al(III), Fe(II) (Table I), is the order of increasing binding constant.

The high affinity of conalbumin and transferrin for aluminum suggests that the transferrins may serve as carriers for the biological transport of aluminum as well as of iron. Although Al(III) has been reported to be both a competitive and uncompetitive inhibitor of ferroxidase (ceruloplasmin) activity (16), it has not been reported to be a competitive inhibitor of iron transport to reticulocytes, perhaps because it has never been tested.

An increasing amount of evidence has been presented to demonstrate that the two metal binding sites of conalbumin and transferrin are not identical. Two of the largest rare-earth ions, Nd(III) and Pr(III), are bound to only one of the metal binding sites of human transferrin (16). Similar results were obtained with conalbumin. Characteristic changes in the EPR spectrum of conalbumin and transferrin as a function of perchlorate ion concentration were interpreted as direct evidence for a distinction between the metal binding sites (17). Significant differences have been demonstrated in the EPR, Mössbauer, and optical spectra of conalbumin when the two binding sites were filled with different metals or isotopes of iron in a particular order (18). However, the circular polarization of luminescence of terbium ions bound to transferrin and to conalbumin (19) indicates that the two metal-binding sites are equivalent in structure and conformation. Our present demonstration that the first Al(III) bound to conalbumin increases its heat stability, but that the second Al(III) bound has no effect on the heat stability, taken together with the (at least) 100-fold difference in binding constants for Al(III), is further evidence that the metal binding sites on conalbumin are not equivalent.

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Note Added in Proof—Professor Paul Saltman has pointed out to us that displacement of Al(III) by Fe(II) (Fig. 3) could be complicated by differential rates of oxidation of Fe(II) to Fe(III) (see Refs. 8 and 9). At his suggestion, we have repeated the displacement experiment with Fe(III)-nitrilotriacetate (20, 21) in place of Fe(II). Qualitatively, the results are the same, but the observed rates are different. Displacement of Al(III) by Fe(III)-nitrilotriacetate, as measured by $A_{440}$, occurs in two equal steps which obey simultaneous first order kinetics. For the faster step, $t_{1/2}$ is 36 s; for the slower, $t_{1/2}$ is 1100 s.

REFERENCES

Nonequivalence of the metal binding sites of conalbumin. Calorimetric and spectrophotometric studies of aluminum binding.

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