# The Stability Constants Of The $Fe^{3+}$ Conalbumin Complexes

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The conalbumins and transferrins are remarkably similar proteins with respect to their molecular size (1, 2, 3), metal binding capacities (4, 5), chromogenic properties (6), and electron paramagnetic resonance spectra (5, 7). Indeed, in the chicken, transferrin isolated from blood plasma and conalbumin isolated from egg white are said to be identical except for their carbohydrate moieties (8). One seeming difference between these proteins is in the ratio of the stability constants of their $Fe^{3+}$ complexes. Each protein specifically and reversibly binds $2Fe^{3+}$ ions. In conalbumin, $K_2$ for this binding has been reported to be 100 times greater than $K_1$ (4), while in transferrin the two constants are found to be essentially equal (5). Because of this apparent and striking functional dissimilarity, we have reinvestigated the stability

constants of the iron-conalbumin complexes by the method of equilibrium dialysis.

Materials and methods: Recrystallized conalbumin purchased from the Nutritional Biochemicals Company was further purified by column chromatography using DEAE Sephadex (8). The final preparation showed only a single component by electrophoresis on cellulose acetate strips (Veronal buffer, pH 8.6). Apoconalbumin was prepared from the iron-bearing protein as previously described (8). Thiomerosal, 1:100,000, was added to all solutions as a preservative.

Equilibrium dialysis was carried out at 25°C (±0.5°) according to the procedures of Aasa et al. (5). To ensure that equilibrium has been achieved, dialysis was continued for 5 days; the results at the end of this time were identical with those obtained after 4 days. Citrate was chosen for the competing chelating agent (5). A citrate concentration of $3^{x}10^{-3}$M and a sodium nitrate concentration of 0.1M was present in both sides of all cells, so that ionic strength was constant throughout the experiments. The desired variation in the average number of $Fe^{3+}$ ions bound to conalbumin was then achieved by varying the total amount of $Fe^{3+}$ present in each cell. $Fe^{3+}$ was added as $^{59}FeCl_3$, and assayed with a scintillation counter. The experiments were carried out in a well-ventilated room, and the $CO_2$ content of the atmosphere in equilibrium with the cells was assumed to be 0.036%. A variation in this figure would proportionately change the value

of the final equilibrium constants without, however, changing the shape of the corresponding experimental curves (Figure 1).

Results: A computer program was utilized for the mathematical analysis of experimental data, following the treatment given by Aasa et al, (5).

In this treatment $K_2$ is set equal to $\frac{1}{4} RK_1$, where $\frac{1}{4}$ is the statistical factor for the binding of 2 metal ions and R is an adjustable parameter which will have a value of unity when $K_1$ and $K_2$ are intrinsically equal. The equations for the

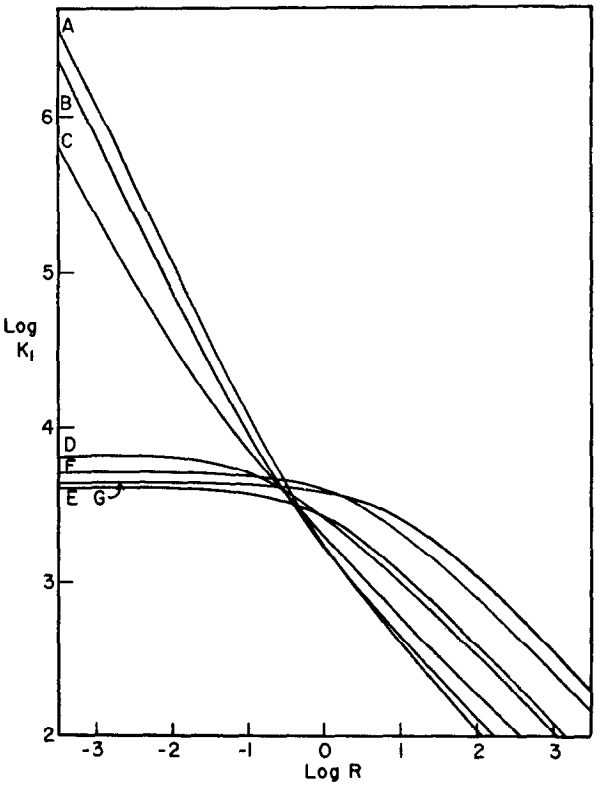


Fig. 1 The calculated dependence of K1 on the adjustable parameter R. Each curve represents a single equilibrium dialysis experiment. The best value of R is taken as that where the ratio of the root mean square deviation of K1 to the mean value of K1 is a minimum. Curves labelled as in Table I.

TABLE I

| Expt. | Concentration of $Fe^{3+}$ ($\mu$M) at Equilibrium | | pH | | Average number of $Fe^{3+}$ bound per molecule of conalbumin | $-\text{Log}[Fe^{3+}]$ | $K_1$ (R=0.3) |
|---|---|---|---|---|---|---|---|
| | Protein Comp. | Non-Protein Comp. | Protein | Non-Protein | | | |
| A | 66.8 | 28.7 | 6.66 | 6.66 | 1.46 | 18.1 | 4800 |
| B | 55.6 | 21.0 | 6.67 | 6.66 | 1.33 | 18.3 | 4300 |
| C | 55.5 | 22.2 | 6.65 | 6.64 | 1.28 | 18.2 | 3800 |
| D | 42.7 | 15.2 | 6.61 | 6.60 | 1.05 | 18.4 | 3600 |
| E | 17.3 | 30.0 | 6.66 | 6.66 | 0.55 | 19.1 | 3300 |
| F | 9.25 | 0.49 | 6.75 | 6.77 | 0.34 | 20.0 | 5200 |
| G | 4.85 | 0.41 | 6.68 | 6.67 | 0.17 | 20.0 | 4000 |
| | | | | | | Average | 4100 |

calculations of $K_1$ are taken from Aasa et al, (5), and are essentially equivalent to those used by Warner and Weber (4).

The data from these equilibrium dialysis experiments are presented in Table I, and the results of the calculations are shown in Figure I. From the graphical display it is clear that the value of R which best fits the data is near 0.3 so that the intrinsic binding constants $K_1$ and $K_2$ are then 4100 and 310 respectively.

Discussion: The present results are consistent with earlier electrophoretic studies from this laboratory and from Warner's laboratory in which 3 species of conalbumin were found (1, 8). These were identified as apoconalbumin, Fe-saturated conalbumin with $2Fe^{3+}$ bound per molecule, and conalbumin with 1 bound $Fe^{3+}$ Since all 3 species were found to coexist at equilibrium, it was concluded that $K_2$ could not be much greater than $K_1$. These earlier studies, and the present report, are in conflict with other measurements by Warner and Weber, who reported $K_2 >> K_1$ (4). Several possible explanations may account for this discrepancy. In the work of Warner and Weber only 16 to 24 hours were allowed for the attainment of equilibrium; Aasa et al, subsequently showed that several days were probably necessary (5). The variations in ionic strength resulting from the variations in citrate concentration may have had some effect on the binding constants. Our own experience would support this view since we have obtained satisfactory convergence of the equilibrium curves only when

ionic strength was held constant. Initial binding of $Fe^{3+}$ to conalbumin may occur without color formation (10); if so, determination of bound $Fe^{3+}$ on the basis of color developed may have led to erroneous results in the earlier work. Finally, there is some evidence that the chemical configuration of the metal-binding sites of transferrin undergoes a transformation below pH 6·5, and a similar change may occur in conalbumin (11). Since a number of the experiments upon which the calculations of Warner and Weber are based were carried out below pH 6·5, this possibility is not excluded.

The close relationship between conalbumin and transferrin is further corroborated by the present findings.

Although the absolute values of the stability constants of the iron complexes are somewhat greater in conalbumin, the observed variations may be due to differences in experimental technique. It seems clear, however, that there is no great interaction between the metal-binding sites of conalbumin. The binding of $Fe^{3+}$ to this protein may then be described as coordination to two equivalent sites (7, 10) displaying a weak negative interaction.

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