The Interaction of Iron-Conalbumin(Anion) Complexes with Chick Embryo Red Blood Cells*

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SUMMARY

Equilibrium binding studies and iron transport rate studies of the interaction of conalbumin and transferrin with the chick embryo red cell have supported the following conclusions: (a) that the chick embryo red cell is comparable to the rabbit and human reticulocyte systems traditionally used for the study of the physiological function of the metal complexes of siderophilins; (b) that there may be two classes of binding sites on the chick embryo red cell membrane for Fe₂conalbumin, one for which conalbumin competes and one for which it does not compete; (c) that the red cell membrane accepts iron from different conalbumins at twice the rate as from monoferriconalbumins, suggesting that simultaneous transport takes place from the two iron-binding sites on the protein with respect to iron acquisition; and (d) that the substitution of oxalate for carbonate as the obligate anion in the Fe₂conalbumin( anion) complex decreases the rate of iron uptake by the cell because the cell must release the anion from the protein complex before it is able to sequester the bound iron.

Since the report of Walsh et al. (1) that the anucleate erythroid cell, the reticulocyte, is the circulating cell responsible for the acquisition of transferrin-bound iron required for the synthesis of hemoglobin, several groups of investigators have carried out fundamental studies describing the interaction of iron-transporting proteins with reticulocytes.

Jandl et al. (2) studied human reticulocytes from patients with severe anemia and established that iron transport from transferrin is a function of cellular oxidative metabolism and of pH and temperature values within the physiological range, and that iron is not freely available to chelators such as EDTA during the transport process. Schade (3) and Morgan and Laurell (4) expanded these studies to include the rabbit reticulocyte-transferrin system, and demonstrated that the process of iron transport is saturable with respect to the metalloprotein complex. Morgan and his co-workers (5–8) have refined the rabbit reticulocyte studies to define the kinetics and equilibria of binding of the metalloprotein to the cell and the metabolic requirements for the transport of iron to the cell.

Although there is a considerable stock of physical and chemical data available regarding the transition metal complexes of siderophilins, particularly transferrin and conalbumin, no consensus has yet been reached with respect to the mechanism of iron binding to the protein or the mechanism of iron release from the metalloprotein complex to the reticulocyte. Arguments have been presented to support the concept that the two metal-binding sites are equal and independent (9, 10) and, on the contrary, that iron binds to the two sites in a cooperative manner (11, 12). It now appears that the two iron-binding sites differ as revealed by their spectra (13, 14). With respect to the release of iron from the protein to the reticulocyte, Schade (3) suggested cooperative release while Fletcher and Huehns (15, 16) proposed that the reticulocyte accepts one iron from iron-transferrin more readily than the other. Morgan and Appleton (8) have presented autoradiographic data which suggest that the reticulocyte takes up the entire metalloprotein complex rather than the iron atoms alone.

The resolution of such conflicting information requires not only an understanding of the internal relationship of the metal-binding sites on the protein, but also delineation of their physiological relationship. To this end, the chick embryo red cell system used in the present study was developed. This cell system is convenient and reliable, and data obtained from it are intended to complement data obtained from the reticulocyte systems currently in use.

EXPERIMENTAL PROCEDURES

All solutions and dilutions were made with glass-distilled water. Chemicals were reagent grade and were used without further purification.

Conalbumin and Transferrin Preparation—Conalbumin from hen's egg white was prepared by the method of Woodworth and Schade (17) with the exception that the protein was chromatographically purified. We adopt "siderophilin" as the class name for all reversible iron-binding globulins found in biological fluids of animal origin and restrict the specific names to particular proteins, e.g. conalbumin is the siderophilin from egg white, transferrin is the siderophilin from blood plasma. This usage was suggested by Philip Aisen.
graphed on CM-Sephadex C-50 instead of on CM-cellulose. Chicken transferrin was prepared from hen and cock plasma by a modification of the conalbumin preparation which employed isoelectric focusing (18) to separate transferrin from contaminating conalbumin. Both proteins were stored in the iron-free state at -20°C.

The iron complexes of conalbumin and transferrin were prepared by adding the appropriate amount of standard 20 mM ferric perchlorate (G. F. Smith Chemical Co.) to the protein dissolved in buffered saline (0.15 M NaCl, 20 mM NaHCO₃). For iron-transport rate studies, \(^{59}\text{FeCl}_3\) (New England Nuclear) or \(^{59}\text{FeCl}_3\) (Amer sham-Searle) was diluted with the standard ferrous perchlorate solution to obtain the desired specific activity. Iron binding was measured from the characteristic absorbance of the iron-conalbumin complex at 465 nm. Spectral data for conalbumin have been summarized (17), and the same values were used for chicken transferrin since it differs from conalbumin only in its carbohydrate content (10). Mixed isotope and mixed metal complexes of conalbumin were prepared as previously described (14). Metalloconalbumin complexes containing anions other than carbonate were prepared by adjusting the conalbumin solution to pH 6 in a Thunberg cuvette, adding the anion solution to a final concentration four times that of the conalbumin, and placing enough metal ion solution in the bulb nearly to saturate the conalbumin. After evacuation (aspirator) to remove CO₂, the metal ion solution was tipped in and mixed, and the pH was raised to approximately 8 by addition of NH₄OH gas through a rubber septum in the sidearm. About 0.5 ml of air was also admitted to provide sufficient oxygen to oxidize the ferrous ion to the ferric state in the protein complex. Spectra were taken, then the complexes were dialyzed versus CO₂-free water to remove ammonium ion, which is inhibitory to the cells. Spectra taken after dialysis showed the characteristic colors of the complexes to be stable and nondialyzable. Iron-saturated conalbumin and transferrin were trace-labeled with \(^{125}\text{I}\) by the method of McFarlane (20) at a calculated level of 1 iodine atom per protein molecule, for equilibrium binding studies. \(^{125}\text{I}\)-labeled solution was obtained from New England Nuclear.

**Chick Embryo Red Cell Preparation**—Red blood cells were taken from white leghorn embryos after 14 to 16 days incubation at 37°C and 80% relative humidity. Embryo wet weights at this stage of development averaged 10 g.

The shell, covering the air space, and the choioallantoic membrane were removed, and the major extracellular blood vessels were severed. The resulting suspension of chick embryo red cells in allantoic fluid was removed from the egg and diluted with a modified Earle's salts solution (21) containing 0.12 M NaCl, 5 mM KCl, 1 mM NaHPO₄, 1 mM MgSO₄, 1 mM CaCl₂, 6 mM glucose, and 2.5 mg per ml of bovine serum albumin (Fraction V, Sigma Chemical Co.). The cells were centrifuged at 700 \(\times g\) and 4°C, then washed twice by resuspending in cold diluent, and recentrifuging. The resulting red cell pellet was very lightly packed. A standard curve of hemocytometer counts on serial dilutions of the cells plotted against hematocrits determined on the same dilution series allowed cell counts to be made for individual experiments by simply determining the hematocrit of the reconstituted blood used in the experiment. Hematocrits were measured in capillary tubes.

**Metal Acquisition Rate Studies**—One volume of the washed red cell pellet described above was added to 2 volumes of Earle's salts solution which had been made 20 mM in NaHCO₃, giving a nominal hematocrit of 20% (1.4 \(\times 10^8\) cells per ml). The suspension was equilibrated with respect to temperature and pH by gentle shaking in a 37°C water bath for 15 min under a continuously flowing atmosphere of 95% air-5% CO₂.

After equilibration, \(^{59}\text{Fe}\)-labeled conalbumin or transferrin was added to give the desired experimental protein concentration. Zero time in the rate determination was taken just after adding and mixing the labeled protein with the cells. Incubation was continued as above.

Sampling for a rate study was carried out at appropriate time intervals by removing an aliquot of the cell suspension and immediately diluting it 10-fold with cold Earle's salts solution. The cells were centrifuged rapidly and washed twice with additional cold suspending medium. The radioactivity contained in the washed red cell pellet was determined in a well-type scintillation counter. Hematocrit and pH determinations were made on each reaction mixture at the conclusion of an experiment.

**Metal and Anion Release Studies**—Incubation mixtures were made up as described above, but disappearance of radiolabeled metal ions and anions from the conalbumin complex was followed by counting a 50- or 100-ml aliquot of supernatant fluid in a Beckman LSC 250 liquid scintillation counter, rather than by counting the washed cell pellet. For each sample 10 ml of a toluidine solution, containing 0.4% 2-(4'-t-butylphenyl)-5-(4'-biphenyl)-1,3,4-oxidiazole fluor, 5% Biosolve-3 (Beckman), and 0.4 mM EDTA was used. With this approach disappearance of \(^{59}\text{Fe}\) and \(^{59}\text{Fe}\), or \(^{59}\text{Fe}\) and \(^{14}\text{C}\) anion could be followed in the same experiment. The \(^{59}\text{Fe}\) isotope emits a low energy y-ray which can be efficiently counted by this method. By using a variable Iso-set module in the LSC 250 and sacrificing the most energetic 20% of the \(^{59}\text{Fe}\) counts, we were able to limit the interference from \(^{14}\text{C}\) or \(^{59}\text{Fe}\) to 6 and 11% of their respective counts. In the anion exchange studies the incubation mixture was placed in a dialysis sac mounted on a rapid dialysis rack (22) and dialyzed against Earle's salts solution containing 20 mM NaHCO₃. The pH was maintained by bubbling 5% CO₂-95% air or 5% CO₂-95% N₂ through the medium, and the whole apparatus was housed in a 37°C water bath. As the anion was released, it dialyzed into the outside medium. Disappearance of \(^{59}\text{Fe}\) and \(^{14}\text{C}\)oxalate was followed as described above by sampling at appropriate intervals the contents of the dialysis sac, by attaching a syringe to a fine plastic tube inserted into the top of the dialysis sac and reaching to the bottom.

**Equilibrium Binding Studies**—Scatchard equilibrium binding studies (23) were carried out in the following manner. Red cells and suspending medium with bicarbonate were combined in the proportions described above. \(^{59}\text{I}\)-labeled protein was added and allowed to establish a binding equilibrium with the cells under the appropriate conditions of pH and temperature (15 to 30 min). The amount of protein bound to the cells at equilibrium was determined by layering an aliquot of the reaction mixture onto a convenient volume of cold, isotonic sucrose (0.200 ml aliquot, 7 ml of 0.25 M sucrose) in a centrifuge tube. The cells were centrifuged through the sucrose column, leaving the interstitial fluid containing unbound radioactive protein at the surface of the column. In the absence of red cells, no radioactive protein penetrated the sucrose column during centrifugation. Protein bound per cell was determined from the radioactivity of the pellet and the number of cells in the original aliquot. Hematocrit and pH measurements were also made on each equilibrium mixture. The concentration of free protein in the suspending medium at equilibrium was determined by centrifuging a separate, undiluted aliquot of the reaction mixture and measuring the radioactivity of the protein in the supernatant fluid.
RESULTS AND DISCUSSION

From a Scatchard plot of the binding of $^{125}$I-Fe$^{2+}$conalbumin to the chick embryo red cell (Fig. 1), the average number of binding sites per cell, $n$, at equilibrium is $2 \times 10^5$ with an apparent binding constant of $1 \times 10^6$ M$^{-1}$ with respect to protein. Baker and Morgan (6) have reported similar values $(3 \times 10^5$ per cell, $1 \times 10^6$ M$^{-1}$) for the rabbit reticulocyte transferrin system. Fig. 1 also demonstrates the lack of significant binding of $^{125}$I-Fe$^{2+}$conalbumin to the adult chicken red blood cell. In a similar study, the binding sites for $^{125}$I-Fe$^{2+}$transferrin were determined to be $1 \times 10^5$ per cell, with an apparent binding constant equal to that for conalbumin. The value of $n$ has been observed to vary within a factor of 2 from one cell preparation to another. The basis for this variability is not presently understood.

Fig. 2 is a Scatchard plot comparing the binding of $^{125}$I-Fe$^{2+}$conalbumin to the chick embryo red cell as a function of the anion bound in the metalloprotein complex. The binding parameters of $^{125}$I-Fe$^{2+}$conalbumin(oxalate)$_2$ and $^{125}$I-Fe$^{2+}$conalbumin(glycinate)$_2$ are similar to those for the $^{125}$I-Fe$^{2+}$conalbumin(carbonate)$_2$ control. However, rate studies discussed below indicate that bound oxalate is capable of inhibiting the delivery of iron from conalbumin to the chick embryo red blood cell.

The effect of competing, iron-free conalbumin on the observed value for the Scatchard parameter $\beta$ is described by Fig. 3. The average number of protein molecules bound per cell at equilibrium is plotted with respect to the ratio of [conalbumin] to $[^{125}$I-Fe$^{2+}$conalbumin]. $^{125}$I-Fe$^{2+}$conalbumin was held constant at 6 $\mu$m, and $\beta$ was determined for that species in the presence of 6 to 190 $\mu$m conalbumin. The distinct departure from linearity as values of $\beta$ decrease in response to increasing conalbumin concentration suggests that there is a class of binding sites for $^{125}$I-Fe$^{2+}$conalbumin on the red cell membrane for which the metal-free protein successfully competes, as well as a class of sites for which the metalloprotein has a greater affinity. No Scatchard studies were performed with the iron-free protein because iron bound to conalbumin could not be completely removed subsequent to trace iodination.

Metal Acquisition Rate Studies The chick embryo red cell system was found to be comparable to reticulocyte cell systems with respect to metabolic control of the metal transport rate. The optimum pH range was 7.2 to 7.4, a result in agreement with that reported by Jandl et al. (2) for a human reticulocyte-transferrin system. Low temperatures, hypoxic conditions, and inhibitors of oxidative metabolism such as 2,4-dinitrophenol also severely inhibit the rate of iron transport from Fe$^{2+}$conalbumin to the chick embryo red cell. Comparable data have been reported for the rabbit reticulocyte-transferrin system (7).

Given optimum metabolic conditions for the transport of iron, the rate may be limited by the relative concentrations of membrane-binding sites and reacting protein. Since iron transport from iron-siderophilin to the reticuloocyte is dependent on the formation of an equilibrium complex, the rate of iron uptake by the cell will be a function of the metalloprotein concentration whenever it is too low to saturate the membrane-binding sites.

In practical terms, reticulocytes incubated with metallo siderophils for extended periods, such that the siderophilin becomes depleted of metal, may show rate limitation owing to depletion of substrate. Lowered iron-transport rates under these circumstances may be misinterpreted unless it is shown that substrate rate limitation is not occurring. Fig. 4 illustrates a determination of the Fe$^{2+}$conalbumin concentration required for iron-transport rates independent of substrate concentration in the chick embryo system. $^{55}$Fe-saturated plasma was incubated with two levels of chick embryo red cells so that the hematoctrits were 25 and 50%. The decrease in iron uptake after 90 min is due to depletion of substrate in the mixture containing the greater number of cells. At this point, the Fe$^{2+}$conalbumin concentration was approximately 7 $\mu$m. In all experiments where metal transport rates were compared, care was taken to exceed this concentration.

Fletcher and Huehns (15, 16) have described experiments with a reticulocyte-transferrin system which show a decrease in the rate of iron transport from protein depleted of iron by prior incubation with reticulocytes, as compared with an untreated control. They interpret their observations as indicating that the young red cell accepts iron more readily from one iron-

![Fig. 1 (left). Scatchard plot of the binding of $^{125}$I-Fe$^{2+}$conalbumin to chick embryo red cells (•), and to adult hen red cells (○). Protein concentration range was 0.1 to 7 $\mu$m.](image1)

![Fig. 2 (center). Scatchard plot of the binding of $^{125}$I-Fe$^{2+}$conalbumin(anion)$_2$ to chick embryo red cells as a function of the anion bound in the metalloprotein complex. Anions: carbonate (•), glycinate (□), and oxalate (○). Protein concentration range was 0.1 to 6 $\mu$m.](image2)

![Fig. 3 (right). Effect of increasing conalbumin concentration on the level of binding of $^{125}$I-Fe$^{2+}$conalbumin to chick embryo cells. $^{125}$I-Fe$^{2+}$conalbumin concentration was 6 $\mu$m.](image3)
Fig. 4. Rates of iron uptake by chick embryo red cells from $^{59}$Fe-saturated hen plasma as a function of the relative concentrations of cells and available iron. Hematocrits: 0.25 (●) and 0.50 (○).

Fig. 5. Effect of removal of iron from $^{59}$Fe-conalbumin by chick embryo red cells on the ability of the metalloprotein to donate iron to fresh chick embryo red cells. Times of preincubation and concentration of bound iron at start: control, 0 min, 30 μM; A, 30 min, 24 μM; B, 60 min, 22 μM; C, 90 min, 19 μM; and D, 120 min, 17 μM.

Fig. 6. Rates of $^{59}$Fe uptake by chick embryo red cells from the following species: upper set of rates, $^{59}$Fe-conalbumin(p1) plus 1 eq of $^{59}$Fe (●), $^{59}$Fe-conalbumin(p1) (○), and $^{59}$Fe-conalbumin (control) (■); lower set of rates, $^{59}$Fe-conalbumin(p1) (○), $^{59}$Fe-$^{59}$Fe-conalbumin (A), Cr,$^{59}$Fe-conalbumin (○), Ga,$^{59}$Fe-conalbumin (●), and $^{59}$Fe,Ga,$^{59}$Fe-conalbumin (▲). p1 indicates species prepared by isoelectric focusing.

binding site on the protein than from the other iron-binding site.

Fig. 5 illustrates a duplication of Fletcher and Huehn's general experimental design in the chick embryo red cell system. The iron uptake rate measured over the full 2-hour period is the control system with an initial Fe-conalbumin concentration of 15 μM. At successive 30-min intervals, an aliquot of the control mixture was removed and centrifuged to separate the red cells. The supernatant fluid containing iron-depleted conalbumin (82, 73, 64, and 58% iron-saturated at each successive 30-min sampling period) was resuspended with fresh chick embryo red cells and the rate of iron transport again determined over a 40-min period. The rates of iron donation from each of the iron-depleted protein samples were the same as the control.

In view of recent spectroscopic data which indicate that the two iron-binding sites of conalbumin differ (14), a straightforward determination of the relative rates of iron release to the red cell from those sites is imperative. The one-iron conalbumin species, Fe-conalbumin, and various mixed metal-conalbumin species were assayed for their iron-donating ability, as summarized in Fig. 6. The rates of iron transport into the chick embryo red cell from the untreated control species, Fe-conalbumin, the same species isolated by isoelectric focusing, and $^{59}$Fe-conalbumin to which 1 eq of $^{59}$Fe had been added were similar. Showing half the transport rates of the doubly-labeled proteins were Fe,$^{59}$Fe-conalbumin, Fe,$^{59}$Fe,$^{59}$Fe-conalbumin, Ga,$^{59}$Fe-conalbumin, Cr,$^{59}$Fe-conalbumin, and Fe,Ga,$^{59}$Fe-conalbumin. These results suggest that the chick embryo red blood cell removes whatever iron it finds on a conalbumin molecule and does not discriminate between the iron bound at the two different sites. Occupancy of the other site by a nonacquired metal ion does not affect the acquisition of the protein-bound iron by the cell.

The nomenclature for mixed metal and mixed isotope complexes of conalbumin is that previously described (14). Metal ions specifically bound to the protein are prefixed to conalbumin with a positional subscript, namely Fe,conalbumin means monoferriconalbumin with the iron bound in the "inner" or first-added site, and Ga,Fe,conalbumin means monogalliummonoferriconalbumin with iron bound in the inner or first-added site, and gallium bound in the "outer" or second-added site.
FIG. 7. Comparison of relative rates of release of 59Fe and 55Fe from 59Fe-55Fe-conalbumin to chick embryo red cells. The rates are expressed as the fraction released per min ± s (standard deviation of the rate). The drawn line represents the average rate for all points. For the separate isotopes and two different protein preparations, the rates ± s were: (from freshly prepared conalbumin), ○, 59Fe, 0.0033 ± 0.0005; (from 6-month-old conalbumin), ●, 59Fe, 0.0034 ± 0.0004; ○, 55Fe, 0.0022 ± 0.0005. In both cases the hematocrit was 0.30 (2.0 × 10^10 cells per ml), and the initial FeZconalbumin concentration was 24 μM.

The ultimate test of the relative rates of release of the two conalbumin-bound ferric ions to chick embryo cells was made by studying the disappearance of 59Fe and 55Fe from 59Fe-55Fe-conalbumin, produced by adding 1 eq of 59Fe to 55Fe-conalbumin isolated by isoelectric focusing. Fig. 7 shows the results of two experiments with freshly isolated and 6-month-old conalbumin preparations. Evidently the cells acquire iron at essentially equal rates from the two differentially labeled sites on diferric conalbumin. Analysis of the final supernatant solution from these uptake studies by isoelectric focusing revealed that the solution contained approximately equal amounts of conalbumin, FeZconalbumin, and Fe55conalbumin, and that the 59Fe and 55Fe labels were equally distributed in both the one-iron and two-iron conalbumin species. Similar results were obtained with control incubation mixtures containing 59Fe-55Fe-conalbumin and conalbumin but no cells. These findings suggest that under the incubation conditions, or during the isoelectric focusing procedure, the following exchange process occurs:

\[ 59\text{Fe},55\text{Fe-conalbumin} + \text{conalbumin} \rightarrow 59\text{Fe-conalbumin} + 55\text{Fe-conalbumin} \]

The experiments performed to date do not enable us to decide at what stage this exchange process occurs, but at least they indicate that it does not require the presence of iron-acquiring cells.

It is clear from the foregoing data that the chick embryo red cell is able to accept simultaneously iron bound to the two sites on conalbumin. This finding is at variance with the Fletcher-Huehns hypothesis (15, 16). Whether this discrepancy lies in a difference between the physiological functioning of conalbumin and transferrin or in a difference between the iron-acquiring mechanisms of chick embryo red blood cells and rabbit reticulocytes cannot at present be decided.

In the iron-uptake experiments described thus far, the natural physiological anion, carbonate, has been involved. The physiological effect of the anion obligately bound in the iron-conalbumin complex was investigated by studying the rates of iron uptake by chick embryo red cells from a series of FeZconalbumin(anion)2 complexes. These complexes have distinct, characteristic visible spectra (Fig. 8), and are stable to dialysis against CO2-free water. The rates of iron uptake were similar for complexes in which the bound anion was carbonate ( ), glycolate ( ), glyoxalate ( ), glycine ( ), thio glycolate ( ), salicylate ( ), and oxalate ( ).

The question then is whether bound oxalate allows the selec-
plexes were made with a stoichiometric amount of iron or gallium, but with a 2-fold molar excess of oxalate to assure rapid and complete formation of the complexes. Without cells: SS~ (0) and [14C]oxalate (A) from the iron complex, and [14C]oxalate (m) from the gallium complex. With cells (aerobic): 55Fe (0) and [14C]oxalate (A) from the iron complex, and [14C]oxalate (m) from the gallium complex.

In the presence of one oxalate for carbonate in the metalloconalbumin(oxalate)~ complex; and (c) exchange, or nonexchange, of the second oxalate can be resolved into three straight lines representing, in decreasing order (a) washout of excess, nonbound oxalate; (b) exchange of one oxalate for carbonate in the metalloconalbumin(oxalate)~ complex; and (d) exchange, or nonexchange, of the second oxalate in the complex. In the absence of cells both metalloconalbumin(oxalate)~ species exchanged one oxalate for carbonate and retained one oxalate. This result is akin to that found for the Fe-transferrin([14C]oxalate)~ complex (25). In the presence of cells, under aerobic conditions, the second oxalate was released at the same rate as iron uptake into the cells. The cells also catalyzed release of the second oxalate from the gallium complex, although in separate uptake experiments we found little or no incorporation of gallium into cells incubated with [56Ga]conalbumin(carbonate)~. Under anaerobic conditions, however, the cells failed to catalyze release of the second oxalate from either the iron or gallium complex and did not take up iron. Evidently anion release, in addition to iron uptake, is dependent on oxidative metabolism. We conclude that the cell probably effects release of the bound anion from the metalloconalbumin(anion) complex prior to release of the metal ion. A protonation step for anion release is a likely possibility, as oxalate, the only inhibitory anion found so far, has significantly lower pK values than the other anions investigated. The anion thus may serve to lock in the bound metal ion, thereby protecting it from competing reactions such as hydrolysis. Such a view is consistent with the finding that iron-transferrin complexes cannot exist in the absence of bound anion (26).

In summary we conclude that the chick embryo red cell does not discriminate between the two ferric ions bound to saturated iron conalbumin, in spite of the demonstrated physical difference between the binding sites, that the cell effects the release of the obligate anion prior to or simultaneously with sequestering the bound iron, and that such anion release requires aerobic metabolism by the cell.

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