Absence of Iron Transfer from Uteroferrin to Transferrin*

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Transfer of iron from native porcine uteroferrin to apotransferrin was investigated using EPR spectroscopy. Purple (oxidized) or pink (reduced) forms of uteroferrin were incubated with porcine or human apotransferrin under conditions of temperature (37 °C) and pH (6.8) approximating those found in the allantoic fluid of the pregnant sow. Studies were also performed in the presence of mediators such as ascorbate, citrate, and ATP in concentrations previously claimed to be effective in promoting large-scale transfer of iron (Buhi, W. C., Ducsay, C. A., Bazer, F. W., and Roberts, R. M. (1982) J. Biol. Chem. 257, 1712–1723). Our experiments indicate that even in the presence of mediators, less than 20% of the iron in uteroferrin is transferred to apotransferrin at the end of 24 h and such transfer may be accompanied by denaturation of uteroferrin. We therefore conclude that the direct transfer of iron to apotransferrin is unlikely to be a physiological role of uteroferrin.

Uteroferrin is an iron-binding single-chain glycoprotein of molecular weight between 35,000 and 40,000 (1–4). It may be isolated in large quantities from either the allantoic fluids of pregnant sows or the uterine flushings of hormone-treated pseudopregnant sows (5–7). The protein can exist in two interconvertible forms: the oxidized or purple form (absorption maximum at 550 nm) which is enzymatically inactive and the reduced or pink form (absorption maximum at 510 nm) which has acid phosphatase activity (2, 3).

It has been estimated that by day 60 of pregnancy in the sow, production of uteroferrin, a maternal protein, may exceed 1 g/day (8). This represents greater than 3 mg of iron, more than the amount transferred from sow to fetuses per day; and thus it has been postulated that uteroferrin plays a key role in providing iron for the fetal pig (8–11). In particular, direct transfer of \(^{59}\)Fe from reconstituted uteroferrin to apotransferrin was observed in vitro, and it has been suggested that the distribution of iron from the allantoic sac to the fetuses occurs via newly iron-bound fetal transferrin (8).

In this study, we investigate further the possibility of in vitro transfer of iron from native uteroferrin to apotransferrin. The reaction was examined for both forms of uteroferrin and two types of apotransferrin (porcine and human) in the presence and absence of ascorbate, citrate, and ATP. EPR spectroscopy was used in monitoring and quantitating the reaction, making use of the \(g = 4.3\) signal of iron specifically bound to transferrin. Our studies obviated using labeled uteroferrin which is stripped of iron under strong reducing conditions and then reconstituted with \(^{59}\)Fe.

MATERIALS AND METHODS

Preparation of Uteroferrin—Uteroferrin was isolated from the uterine flushings of pseudopregnant sows according to published procedures (2, 7). The samples used in these studies had optical purity indices (\(A_{280}/A_{308}\)) of less than 15. Such samples were shown to be homogeneous by sodium dodecyl sulfate-acrylamide gel electrophoresis run under reducing conditions (12). Protein concentrations were estimated from the extinction coefficient at 280 nm of 50 mM \(^{-1} \text{cm}^{-1}\) (13). Pink (reduced) uteroferrin was prepared in accordance with Ref. 14. The purple (oxidized) form of uteroferrin was generated by adding a 20-fold molar excess of hydrogen peroxide to the pink form, followed by chromatography on Sephadex G-75. Such samples displayed residual phosphate levels, as determined by the method of Bartlett (15).

Preparation of Transferrins—Porcine transferrin was purified from pig serum obtained from Pel-Freez according to published methods (18). This procedure produced an electrophoretically homogeneous transferrin band with an absorbance ratio at 470/410 nm of greater than 1.3 (19). The chelate-free apoprotein was prepared by dialysis against citrate, perchlorate, and water (19). A final dialysis was performed versus two changes of HEPES buffer, pH 6.8, followed by concentration to the desired level.

Human transferrin was isolated from pooled blood bank plasma as described in Ref. 18. Only protein samples with an absorbance ratio at 470/410 nm of greater than 1.3 were used. Aporotransferrin was obtained through the sequential dialysis regimen already described for the porcine apoprotein (19), with a final dialysis versus two changes of HEPES buffer, pH 6.8. Aporotransferrin was then concentrated to the desired level.

Reagents—Citrate and l-ascorbate were purchased from J. T. Baker Chemical Co., and ATP from Sigma. Prior to use, all solutions were prepared fresh in HEPES buffer, and the pH was adjusted to 6.8. Ferrous ammonium sulfate was obtained from Mallinckrodt Chemical Works, and was prepared as described in Ref. 21.

To minimize contamination by extraneous metal ions, especially iron, all buffers were treated with Chelex 100, all columns were washed with 0.1 mM EDTA, and all glassware was acid-washed.

EPR Studies—The transfer of iron from uteroferrin to apotransferrin was monitored through the intensity of the EPR spectra of

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‡The abbreviation used is: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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iron-bound transferrin, which were recorded with a Varian E-9 spectrometer interfaced to an Apple II+ microcomputer. Because of line broadening at temperatures above 20 K, the $g' = 1.74$ EPR spectrum of uteroferrin cannot be detected at our operating temperature of 77 K (20). In analyzing the spectra, two measures of signal intensity were used: the peak-to-peak intensity of the transferrin signal and the integrated intensity of the transferrin $g' = 4.3$ line cluster using a program written in Applesoft. The instrumental parameters were: field set, 1500 G; temperature, 77 K; microwave power, 10 milliwatts; microwave frequency, 9.208 GHz; modulation amplitude, 10 G; modulation frequency, 100 kHs; gain, 1.25 x 10^5 or 2.5 x 10^5; time constant, 1 s; and scan time, 8 min.

EPR spectra of iron-saturated transferrin standards were recorded for both porcine (0.54 mM in iron) and human (0.51 mM in iron) proteins at gains of 1 x 10^5 and 1.25 x 10^5, respectively, under the same parameter conditions listed above.

All experiments were run under conditions of temperature (37°C) and pH (6.8) approximating those found in the allantoic fluid of the pregnant sow. The concentration of uteroferrin used was approximately 8 times greater than its physiological concentration (8). In a typical study, equimolar concentrations of uteroferrin (pink, purple, or reconstituted at 100 pM final concentration) and apotransferrin (porcine or human at 100 μM final concentration) in the presence or absence of ascorbate, citrate, or ATP were mixed in a test tube. A zero-time aliquot of 300 μl was immediately taken, inserted into a Wilmad precision EPR tube, frozen in liquid nitrogen, and its EPR spectrum was recorded. The test tube containing the experimental mixture was sealed with multiple layers of parafilm and kept in a constant 37°C water bath. Subsequent aliquots of 300 μl were removed at desired times and processed in a similar manner.

Several control experiments were performed to check the integrity of the proteins under study. As a first measure, the concentration of each protein was determined before and after individual incubation at 37°C and pH 6.8. All proteins had concentration recoveries (defined as: (final concentration/initial concentration) x 100%) of 100% except pink uteroferrin, which had a recovery of 84%.

To determine any effect of freezing (in liquid nitrogen) on the intensity of the porcine transferrin signal, EPR spectra were recorded at 298 K (Fig. 1A) and 77 K (Fig. 1B) for the diferric protein at two concentrations (1.14 mM in iron and 2.28 mM in iron). The ratio of the signal intensity at 298 and 77 K was calculated at one concentration and compared to the same ratio at the other concentration. Upon freezing, we retain 99% of the signal intensity calculated using peak-to-peak analysis (92% using integration). Identical results were obtained using human diferric transferrin.

As a final measure, the amount of apotransferrin available to uteroferrin for iron transfer at the end of 24 h was determined. The 0- and 24-h aliquots of the experiments using no mediators, previously stored in liquid nitrogen, were thawed; and ferrous ammonium sulfate was added directly to the EPR tube in one-half the stoichiometric amount of iron present in uteroferrin. This mixture was kept for 24 h at 25°C, frozen in liquid nitrogen, and the transferrin EPR spectra were recorded. The ratio of the 24-h signal intensity to the zero-h signal intensity was greater than 90% using both peak-to-peak and integration analyses.

**RESULTS AND DISCUSSION**

Using EPR, it was possible to quantitate the percent transfer of iron from native uteroferrin, not subjected to harsh reducing conditions, to apotransferrin. Signal intensities of the transferrin spectra were measured at specific time intervals during the experiment, and these were compared to the signal intensity of a standard diferric transferrin spectrum. The following equation was used:

$$\text{% transfer} = \frac{(x/y)\text{(concentration of TF standard)} \times \text{(gain,)}}{(Fe/UF)\text{(concentration of UF)} \times \text{(gain,)}} \times 100\%$$

where TF stands for transferrin, UF denotes uteroferrin, x is the signal intensity of iron-bound transferrin at a specific time minus the signal intensity of the time 0 aliquot, y is the signal intensity of the standard transferrin, gain, is the gain used in recording the standard transferrin spectrum, and gain, refers to the gain used in the transfer experiment. As noted under "Materials and Methods," x and y were determined by both peak-to-peak and integration methods.

In Table I are listed the percent transfers of iron from uteroferrin to porcine apotransferrin, calculated using the above equation. The discrepancy between the values obtained by peak-to-peak and integration methods can be attributed to a broad base-line drift observed during the actual recording of the spectra. Although the integration analyses resulted in values consistently lower than those obtained through peak-

![Table I](image-url)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Conditions</th>
<th>2 h</th>
<th>6 h</th>
<th>10 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purple</td>
<td>1 mM ATP + 1 mM l-ascorbate</td>
<td>4.03 (1.5)</td>
<td>6.92 (3.4)</td>
<td>9.80 (5.6)</td>
<td>13.84 (7.5)</td>
</tr>
<tr>
<td>Purple</td>
<td>1 mM ATP + 1 mM l-ascorbate</td>
<td>10.90 (4.6)</td>
<td>10.90 (4.6)</td>
<td>15.48 (11.1)</td>
<td>10.90 (4.7) (p*)</td>
</tr>
<tr>
<td>Pink</td>
<td>1 mM ATP + 1 mM l-ascorbate</td>
<td>4.10 (1.9)</td>
<td>5.46 (3.2)</td>
<td>6.83 (4.1)</td>
<td>10.92 (6.1)</td>
</tr>
<tr>
<td>Pink</td>
<td>1 mM citrate + 1 mM l-ascorbate</td>
<td>13.61 (12.5)</td>
<td>8.20 (6.0)</td>
<td>9.11 (6.3)</td>
<td>16.44 (6.7)</td>
</tr>
<tr>
<td>Purple</td>
<td>Reconstituted</td>
<td>1.56 (2.1)</td>
<td>16.44 (6.7)</td>
<td>14.09 (2.1) (p)</td>
<td></td>
</tr>
<tr>
<td>Purple</td>
<td>Reconstituted, 1 mM citrate + 1 mM l-ascorbate</td>
<td>18.79 (7.6)</td>
<td>14.09 (2.1) (p)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*p, precipitation; pp, heavy precipitation.
to-peak analysis, the same conclusions may be drawn from each. The maximal transfer of iron observed was less than 20% in 24 h for both the native and the reconstituted proteins during the time of the experiment. This was true even in the presence of suspected mediators such as ascorbate, citrate, and ATP, previously claimed to be effective in promoting large-scale iron transfer in reconstituted proteins (8). Moreover, the greatest transfer was observed in the presence with reconstituted purple uteroferrin in the presence of citrate and ascorbate (Table I).

Experiments were also repeated using human apotransferrin in place of the porcine apoprotein. The percent transfers of iron were calculated using Equation 1, but with human differric transferrin as a standard. The results were similar to the ones using porcine apotransferrin, again with a maximal transfer of iron of less than 20%. The earlier time course of the transfer phenomenon involving native pink uteroferrin and porcine apotransferrin in the presence or absence of ascorbate or citrate was also examined (Fig. 2). Both ascorbate and citrate increased the amount of iron transferred to apotransferrin in the first 2 h, but the amount of transfer observed was less than 6% and followed no simple kinetic law. Similar results were obtained with native purple uteroferrin.

In all experiments using 1 mM ascorbate, substantial precipitation was observed by the end of 24 h. Although no attempt was made to analyze the nature or the formation of the precipitate, one possibility is the degradation of uteroferrin by hydroxyl radicals generated in the presence of ascorbate, oxygen, and protein-bound iron atoms (21).

Our experiments indicate that the maximum transfer of iron from native uteroferrin to apotransferrin, under conditions thought to be optimal for such transfer, is never more than 20% in 24 h and such transfer may be accompanied by denaturation of uteroferrin. All experiments were performed under conditions of temperature (37 °C) and pH (6.8) approximating those found in the allantoic fluid of the pregnant sow, the concentration of uteroferrin used being much greater than its physiological concentration. Moreover, we can infer from our data involving earlier time scales that this transfer reaction is exceedingly slow. We therefore conclude that the direct transfer of iron to apotransferrin is unlikely to be a physiological role of uteroferrin.

Acknowledgment—We thank Dr. Jack Britt of North Carolina State University for providing fresh porcine uterine flushings.

REFERENCES


FIG. 2. Early time course in the transfer of iron from native pink uteroferrin to porcine apotransferrin. Experiments were performed both in the absence (O) and in the presence of 1 mM ascorbate (O) or 1 mM citrate (□). The percent transfers of iron were calculated using integration analysis and Equation 1.