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Factors Affecting the Adenosine Triphosphate Induced Release of Iron from Transferrin†

Franklin J. Carver and Earl Frieden*

ABSTRACT: The release of iron from transferrin was investigated by incubating the diferric protein in the presence of potential iron-releasing agents. The effective chemical group appears to be pyrophosphate, which is present in blood cells as nucleoside di- and triphosphates, notably adenosine triphosphate (ATP). An alternative structure with comparable activity is represented by 2,3-diphosphoglycerate. Neither 1 mM adenosine monophosphate (AMP) nor 1 mM orthophosphate released iron from transferrin. The ATP-induced iron-releasing activity was dependent on weak acidic conditions and was sensitive to temperature and sodium chloride concentration. The rate of iron release rapidly increased as transferrin was titrated with HCl from pH 6.8 to 6.1 in the presence of 1 mM ATP and 160 mM NaCl at 20 °C. Iron release from transferrin without ATP was observed below pH 5.5. Ascorbate (10^{-4} M) reduced Fe(III), but only after iron release from transferrin by a physiological concentration of ATP. A proposal for the mechanism of iron release from transferrin by ATP and the utilization of reduced iron by erythroid cells is described.

Transferrin is the major vertebrate iron transport protein, having a high stability constant for iron on the order of 10^{24} M^{-1} (Aasa and Aisen, 1968; Aasa et al., 1963). In serum the high avidity of transferrin for iron is especially necessary since ferric ion is insoluble at physiological pH. Iron binding is maintained by six metal ligands for each of two sites per molecule of transferrin (Aisen and Brown, 1977). These ligands include the phenolic group of two-three tyrosine residues, the imidazole group of one-two histidine residues, the anionic ligand site (usually filled by carbonate or bicarbonate), and at least one water molecule (Aisen and Brown, 1977).

When transferrin binds to erythropoietic cells, the binding affinity of the transferrin-iron(III) complex is lowered and iron released for heme synthesis. In order to lower the binding affinity of the transferrin-iron(III) complex it may be necessary to protonate one or more of the iron ligands and/or completely dissociate the carbonate (bicarbonate) from the anionic site. It has been suggested that bicarbonate release is a prerequisite for iron release from transferrin in both in vitro and cell-free systems (Aisen and Leibman, 1973; Egyed, 1973, 1975; Martinez-Medellin and Schulman, 1973).

For several years our laboratory has been interested in problems related to the mobilization of iron from transferrin by immature erythroid cells from the bullfrog, tadpole, and rabbit (James and Frieden, 1975; James, 1976; Carver and Frieden, unpublished results). Recent studies have concentrated on the effects of small molecular weight chelators and reducing agents directly on transferrin (Carver and Frieden, 1977). Of particular interest are the nucleotides which are present as iron ligands (i.e., adenosine and guanosine triphosphates) in reticulocytes and mature red blood cells (Bartlett, 1976a,b; Goucher and Taylor, 1961; Konopka et al., 1969; Konopka and Szotor, 1972). The chemical properties of the ATP-Fe(III) complex have been investigated (Goucher and Taylor, 1965; Neuberg and Mandl, 1949). ATP as well as other potential trivalent chelators, such as citrate, have the capacity to exchange iron between transferrin molecules (Aisen and Leibman, 1968; Donovan et al., 1976; Morgan, 1977) and from transferrin to ferritin (Miller and Perkins, 1969; Mazur et al., 1960). ATP is abundant (~2 mM) in immature and mature red blood cells (Bartlett, 1976a,b; Brown et al., 1972) compared to other potential and effective biological trivalent iron chelators.

The present study was undertaken to determine the role of ATP and other nucleotides in the release of iron from transferrin and the effect of reducing agents on free and protein-bound iron. It was necessary to use weak acidic conditions to evaluate the effects of potential iron-releasing agents on transferrin.

Methods and Materials

Chemicals. All chemicals used were reagent or analytical grade.

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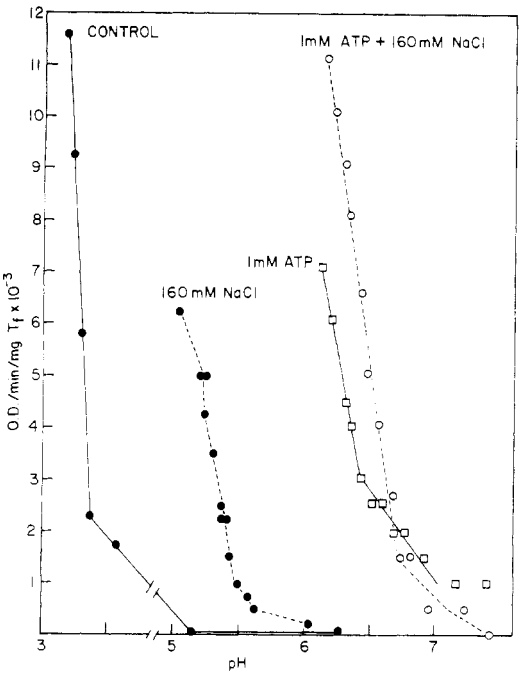


FIGURE 1: The effect of NaCl and ATP on the loss of 465-nm absorption of diferric transferrin as a function of pH. Each reaction was in 20 mM Hepes buffer containing 30 μM transferrin. HCl was added to obtain the desired pH. The total reaction volume was 1 mL. A single point represents the loss of 465-nm absorbing material within the first minute of the reaction at 20 °C; starting OD at 465 nm = 0.13: (●—●) control; (●- - -●) 160 mM NaCl; (□) 1 mM ATP; (○) 1 mM ATP + 160 mM NaCl.

Preparation of Transferrin. Human transferrin (Behringwerke) was prepared by dissolving the protein in 10 mM $NaHCO_3$/150 mM NaCl (pH 7.8) and adding ferric nitrilotriacetate (Fe-NTA_2)[1] to obtain an iron saturation equivalent of 115%. The bicarbonate-transferrin-diferric complex [HCO_3-Tf-Fe(III)$_2$] was allowed to equilibrate at room temperature for 1 h and dialyzed against 20 mM Hepes buffer (pH 7.4) at 4 °C for 3 days with two changes of 20 mM Hepes buffer. The Visking tubing used for dialysis was pretreated with hot 0.1 mM EDTA and then washed with double distilled water at least six times within 2 h and before the addition of the diferric transferrin solution. The concentration of the diferric transferrin was based on absorbance at 465 nm using an $E^{1\%} = 0.57$ and a mol wt of 76 000 (Palmour and Sutton, 1971).

HCl Titration Studies. All nucleotide phosphates for the HCl titration studies were prepared by dissolving in 20 mM Hepes and adjusting the pH to 7.4. The concentrations of the AMP, ADP, and ATP stock solutions were adjusted to 5 mM using an ϵ of 15 400 at 259 nm. The 5 mM stock solutions of the remaining nucleotide triphosphates were based on the following absorption coefficients: ϵ 9000 for CTP at 271 nm, ϵ 9600 for TTP at 267 nm, and ϵ 13 700 for GTP at 253 nm. The inorganic phosphate and pyrophosphate solutions were prepared by weighing sufficient Na_2HPO_4 or $Na_4P_2O_7$ to give a 5 mM final concentration in 20 mM Hepes at pH 7.4.

Prior to each assay the following reagents were added to a quartz cuvette with a 1.00-cm light path: 10 μL hydrochloric acid (0.6–1.0 M to obtain the desired final pH), 590 μL of 20 mM Hepes/271 mM NaCl buffer, and 200 μL of the 5 mM reactant in 20 mM Hepes. The reaction was initiated with 200 μL of transferrin-Fe(III)$_2$ (30 nmol) to give a final volume of 1000 μL and an $A_{465} = 0.13$ ODU (optical density unit). Final component concentrations were 1 mM reactant, 20 mM Hepes or 20 mM Hepes with 160 mM NaCl, and 30 μM diferric transferrin.

Each assay sample was mixed by inverting the cuvette five times and placing it in a Cary 15 spectrophotometer after 15-s elapsed time to record the rate of loss of 465-nm absorbing material (specific for the diferric transferrin complex). The pH of each solution was determined after each assay and found not to vary more than ±0.01 for at least 2 h after the initiation of the reaction with diferric transferrin. The rate, based on the loss of 465-nm absorbing material, was calculated from the first recorded minute (15–75 s) of the reaction using a fast chart speed. Assays were performed at 10, 20, and 30 °C.

Preliminary studies were performed using 30, 50, 70, and 100% iron-saturated transferrin. In the presence of 1 mM ATP the rate of iron release was dependent on iron concentration and independent of the degree of saturation. Since it was necessary to have a reasonably high absorbance at 465 nm and reduce the use of purified transferrin, we conducted all experiments using 100% saturated transferrin.

Studies at pH 6.1. Experiments at pH 6.1 were performed in 25 mM Mes [2-(*N*-morpholino)ethanesulfonic acid]/160 mM NaCl buffer (pH 6.0). All reagents were prepared in double distilled water and adjusted to pH 6.2 with a final concentration of 5 mM reactant and included the following: adenosine triphosphate, sodium pyrophosphate, sodium citrate, 2,3-diphosphoglycerate (P_2-glycerate), nitrilotriacetate (NTA), and ethylenediaminetetraacetate (EDTA). The reducing agents ascorbate and glutathione (GSH) were prepared in water (pH 6.2) at a concentration of 10 mM. Reagents were added in the following order: water and/or 10 mM reducing agent, 5 mM reactant, and 50 mM Mes/320 mM NaCl buffer. Diferric transferrin (30 nmol) in 20 mM Hepes (pH 7.4) was added to initiate the reaction and the rate of loss of 465-nm absorbing material monitored as indicated for the HCl titration studies. The final concentrations of the agents added to the reaction mixture were: 0.1–1.0 mM reducing agent and/or 1 mM releasing agent, 25 mM Mes/160 mM NaCl buffer, and 30 μM diferric transferrin (0.13 ODU at A_{465}). The pH of the reaction mixture for all experiments was 6.1 ± 0.01.

In one study 200 μM 2,2′-bipyridine in water (pH 6.2) was used to monitor the formation of Fe(II) in the reaction mixture. The concentration of the iron(II) was determined by the absorption of the bipyridine-iron(II) complex at 520 nm using an ϵ of 8600. Since transferrin-iron(III) will absorb in the 520-nm region it was necessary to subtract that value from the total 520-nm absorbing material to obtain the final concentration of iron(II) as bipyridine-iron(II).

Results

Effect of pH and NaCl on the Release of Iron from Diferric Transferrin. Preliminary studies using diferric transferrin showed that the loss of 465-nm absorbing material with or without ATP was curvilinear with rapid multiphasic kinetics within the first minute (15–75 s) of the reaction. Figure 1 represents the rates obtained within the first minute by varying the pH of the solution with HCl in the presence of 30 μM diferric transferrin and combinations of 160 mM NaCl and 1 mM adenosine triphosphate (ATP). Each point represents the rate of loss of 465-nm absorbing material within the first minute of the reaction at a given pH. Based on studies to be presented, it was assumed that the loss in 465-nm absorption

[1] Abbreviations used are: Fe-NTA_2, ferric nitrilotriacetate; Tf, transferrin; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; GSH, glutathione; ODU, optical density unit; P_2-glycerate, 2,3-diphosphoglycerate.

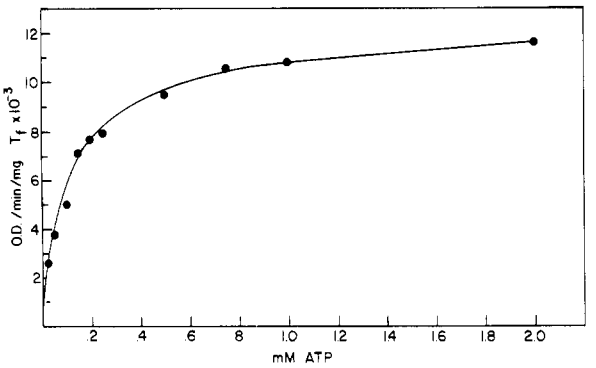


FIGURE 2: The effect of ATP concentration on the loss of 465-nm absorption of diferric transferrin. Reactions were performed in 25 mM Mes/160 mM NaCl at 20 °C, pH 6.1. Total volume equaled 1 mL. Each point represents the loss in 465-nm absorption of 30 μM transferrin within the first minute of the reaction. Starting OD at 465 nm = 0.13.

was due to the release of bicarbonate and iron(III) from the protein.

When diferric transferrin was titrated with HCl in 20 mM Hepes buffer there was no loss of 465-nm absorbing material until pH <5 and the reaction was rapid at pH <4 (Figure 1). The presence of 160 mM NaCl shifted the curve 1.5 pH units to the right at a reaction rate of 2 OD units min^{-1} (mg of Tf)$^{-1}$ × 10^{-3}. Other investigators have titrated transferrin with alkali or acid and found that iron-binding capacity was reduced starting at approximately pH 6 and was at a minimum at pH 5 (Fielding and Ryall, 1970; Lestas, 1976; Surgenor et al., 1949). These results were based on equilibrium studies in which a transferrin solution was adjusted to a given pH and allowed to stand 20 min to 5 days at room temperature or 4 °C; buffer strength varied but was higher than 20 mM. Thus, it is difficult to compare the above with our studies because of different incubation conditions. Our data were collected within the first minute and would correspond to immediate gross changes in protein conformation rather than subtle changes detectable only after hours of incubation. Nonetheless, the curve obtained using 160 mM NaCl was similar to the previously reported data on the effect of hydrogen ion on transferrin iron binding.

In order to observe the effect of ATP on the release of iron from transferrin it was necessary to increase the hydrogen ion concentration. Without 160 mM NaCl the loss in 465-nm absorbing material was rapid and linear at a pH <6.4 and was approximately 2.5 pH units higher than the control curve in which transferrin was titrated with 20 mM Hepes only. The addition of 160 mM NaCl to 1 mM ATP and 30 nmol of diferric transferrin resulted in iron-releasing activity which was highly sensitive to hydrogen ion, and the rate was linear between pH 6.6 and 6.1. The NaCl/ATP curve was displaced upward in pH by 0.2 pH unit compared with the ATP curve, at rate values above 3 ODU min^{-1} (mg of Tf)$^{-1}$ × 10^{-3}.

For all HCl titration curves with diferric transferrin, the observed rate for each set of conditions diminished below a certain pH, e.g., pH 6.1 for 1 mM ATP. This was associated with a rapid loss in absorbancy within the first 15 s of the reaction which was not recorded. Thus, there was a lower concentration of diferric transferrin substrate for the following 60 recorded s and consequently a lower calculated rate (Carver and Frieden, 1977). For clarity, the observations affected by this apparent decrease in rate were not included on the figures in this report.

Effect of ATP Concentration on the loss of 465-nm Absorbing Material. Figure 2 shows that maximum rate of loss

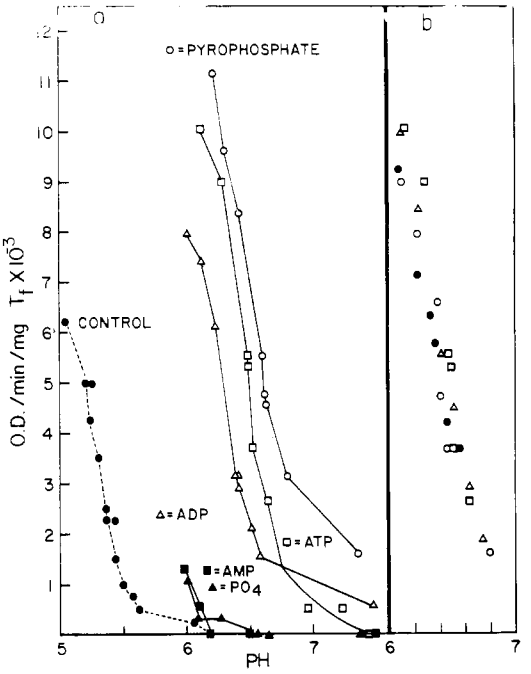


FIGURE 3: (a) The effect of pH on the rate of loss of 465-nm absorbing material from diferric transferrin in the presence of 1 mM adenine ribonucleotides, sodium pyrophosphate, and sodium monohydrogen phosphate: (○) pyrophosphate; (□) ATP; (△) ADP; (■) AMP; (▲) phosphate; (●) Tf only. (b) The equivalence of four nucleotide triphosphates in their effect on the rate of pH-induced loss of 465-nm absorbing material specific for transferrin: (○) CTP; (●) TTP; (△) GTP; (□) ATP. Each reaction was in 20 mM Hepes/160 mM NaCl buffer containing 30 μM transferrin. HCl was added to obtain the desired pH. The total reaction volume was 1 mL. A single point represents the loss of 465-nm absorbing material within the first minute of the reaction at 20 °C. Starting OD at 465 nm = 0.13.

in 465-nm absorbing material at pH 6.1 was achieved at an ATP concentration less than 1 mM, which is within the physiological range in reticulocytes and mature red blood cells (Bartlett, 1976a; Brown et al., 1972).

Effect of Temperature on Iron Release from Diferric Transferrin. HCl titration of transferrin was performed with and without 160 mM NaCl in 20 mM Hepes buffer at 10, 20, and 30 °C. Without NaCl at pH 6.4 the rate at 10 °C was 66% and at 30 °C was 178% of the rate at 20 °C. In the presence of 160 mM NaCl the curves were shifted to the right about 0.10 of a pH unit for each temperature compared to the salt-free curves. At pH 6.4 the rate at 10 °C was 62% of the value at 20 °C. The difference between 20 and 30 °C was not as dramatic as that seen without NaCl and amounted to only 111% of the 20 °C value. Thus, the NaCl appeared to stabilize the transferrin molecule such that temperatures above 20 °C had little additional effect on the release of iron from transferrin.

The Role of Nucleotides on Transferrin Iron Release. Figure 3a represents the effect of pH on the rate of release of Fe(III) from transferrin in the presence and absence of phosphate, pyrophosphate, and the three adenine ribonucleotides. From pH 7.4 to 6.7 the rate of loss of 465-nm absorbing material was minimal. At 1 mM the rates for the following three reactants were rapid from pH 6.6 to 6.2 and were in the following order: pyrophosphate > ATP > ADP.

To determine if the adenosine moiety was specific for the release of Fe(III) from transferrin, 1 mM adenosine, cytosine, guanosine, and thymidine triphosphates were each titrated with HCl in the presence of diferric transferrin (Figure 3b). There was no difference in the rate of Fe(III) release for the four nucleotide triphosphates.

TABLE I: Rate of Loss of 465-nm Absorbing Material Relative to ATP.

Reactant (1 mM)	% of ATP
Pyrophosphate (PP)	105
Adenosine triphosphate (ATP)	100
Guanosine triphosphate (GTP)	100
2,3-Diphosphoglycerate (P_2-glycerate)	95
Thymidine triphosphate (TTP)	92
Citrate	91
Cytidine triphosphate (CTP)	89
Adenosine diphosphate (ADP)	74
Ethylenediaminetetraacetate (EDTA)	73
Nitrilotriacetate (NTA)	36
Adenosine monophosphate (AMP)	5
Phosphate	5
Glutathione (GSH)	4
Ascorbate	0

[a] 30 μM transferrin (0.13 ODU at 465 nm) was incubated in the presence of 1 mM reactant at 20 °C in 25 mM Mes/160 mM NaCl buffer (pH 6.1). The loss of 465-nm absorption was followed for the first minute of the reaction and data converted to a percentage of that obtained for 1 mM ATP. The final volume was 1 mL.

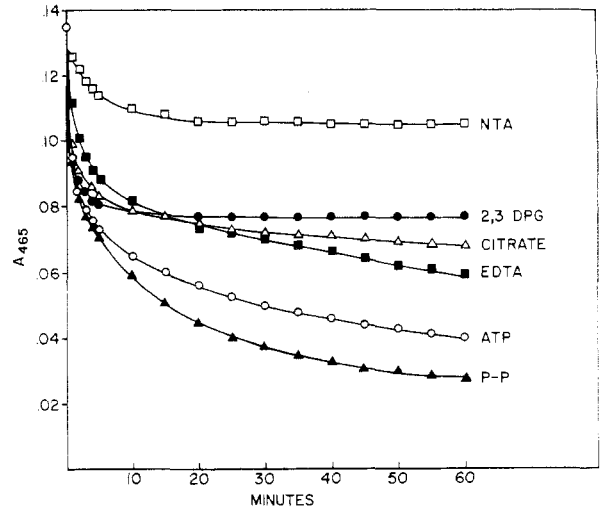


FIGURE 4: A comparison of six potential iron-releasing molecules on the loss of 465-nm absorption over 1 h. 30 μM transferrin (0.13 ODU at 465 nm) was incubated at 20 °C in 25 mM Mes/160 mM NaCl buffer (pH 6.1). The loss of 465-nm absorption was followed for 1 h after the addition of a 1 mM final concentration of each reactant. The total volume was 1 mL: (□) nitrilotriacetate; (●) 2,3-diphosphoglycerate; (△) sodium citrate; (■) ethylenediaminetetraacetate; (○) adenosine triphosphate; (▲) pyrophosphate.

Transferrin–Iron Releasing Potential of Selected Reactants at pH 6.1. Table I lists a number of compounds which have the potential to remove iron from transferrin. These tests were performed at pH 6.1 using 25 mM Mes/160 mM NaCl and the activity was converted to a percentage of that measured for 1 mM ATP. Pyrophosphate was slightly more active than ATP, with GTP, TTP, and CTP having activities slightly lower than ATP. Although ADP was 26% less active than ATP, previous studies (Figure 3) showed it to be much more effective than the control with only transferrin and NaCl. Of interest is 2,3-diphosphoglycerate, containing two phosphate groups, which has an activity slightly less than ATP. P_2-glycerate is found in millimolar concentrations in red blood cells (Bartlett, 1976a) and affects the oxygen dissociation curve of hemoglobin. Citrate, a trivalent chelator, had an activity similar to the nucleotide triphosphates. The reducing agents, glutathione and ascorbate, were not effective in the release of iron from transferrin.

Figure 4 represents the effect of selected reactants on iron release at pH 6.1 over a 1-h period. For all reactants the loss of 465-nm absorbing material was rapid for the first 5 min. NTA and P_2-glycerate were not effective in iron release after 10 min, with NTA at 31% and P_2-glycerate at 43% of the total iron. The loss of almost 50% absorbance in the presence of P_2-glycerate is interesting, since recent studies show that iron and bicarbonate are preferentially loaded and/or unloaded on different iron sites on human transferrin (Aisen et al., 1973; Fletcher and Huehns, 1967; Hahn and Ganzoni, 1975; Hahn et al., 1975; Harris, 1977; Lestas, 1976). Citrate, EDTA, ATP, and pyrophosphate continued to release iron, but at different rates, during 1 h; pyrophosphate was the most active. Loss of absorbance was bimodal for all reactants over the 1-h incubation and suggests a preferential loss of iron from one of the two iron binding sites.

The Effect of ATP and Ascorbate on the Release and Reduction of Iron(III) from Transferrin. This study was designed to determine the ability of ATP to effect iron release and the oxidation state of iron, before and after ATP and ascorbate exposure to 100% saturated transferrin (Figure 5). Reactions were conducted at 20 °C and pH 6.1 using 25 mM Mes/160 mM NaCl buffer. The iron concentration was calculated as 60 nmol in a total volume of 1.00 mL, based on the assumption that two iron molecules were bound to each transferrin molecule.

The control incubation (open squares) represents the release of iron from transferrin at pH 6.1 which, after 90 min, resulted in the release of 3% of the total iron. When 0.1 mM ascorbate was added to 30 nmol of diferric transferrin there was a gradual release of iron which equaled 17% of the total available iron by 90 min (open triangles). A typical iron release curve was obtained when 1 mM ATP was incubated with transferrin (closed circles); there was a rapid loss in 465-nm absorbing material which amounted to 50% of the total iron by 5 min. The ATP–transferrin reaction leveled off by 60 min and effected the release of 77% or 46.2 nmol of the iron by 90 min. The biphasic release of iron from diferric transferrin in the presence of ATP has also been observed by Morgan (1977).

The reaction mixture containing 1 mM ATP and 30 nmol of transferrin was assayed (open circles) with the addition of 0.2 μmol of the Fe(II) chelator 2,2′-bipyridine at 60 min; the latter caused no change in 465-nm absorbing material. There was also no change (data not shown) in 520-nm absorbing material, which is specific for the red color of the bipyridine–Fe(II) complex. Thus, the iron released from transferrin was in the ferric state and possibly complexed with ATP. At 90 min 0.1 mM ascorbate was added to the ATP/transferrin/bipyridine mixture and resulted in an increase in 520-nm absorbing material equaling 42.3 nmol or 92% of the Fe(III) released by ATP and recovered as bipyridine–Fe(II).

In a separate study 1 mM ascorbate was added to a mixture containing 1 mM ATP and 30 nmol of Tf–$Fe(III)_2$. The release of iron was essentially the same as with 1 mM ATP plus 30 nmol of Tf–$Fe(III)_2$ alone. At 60 min 0.2 μmol of bipyridine was added and gave a recovery of 90% of the iron released by ATP in the bipyridine–Fe(II) complex. Glutathione was also found to be an effective reducing agent for ATP-released Fe(III) under the above conditions.

Discussion

The key requirement for the release of iron from transferrin appears to be the pyrophosphate structure which is present as di- and triphosphates in developing erythroid cells. An alter-

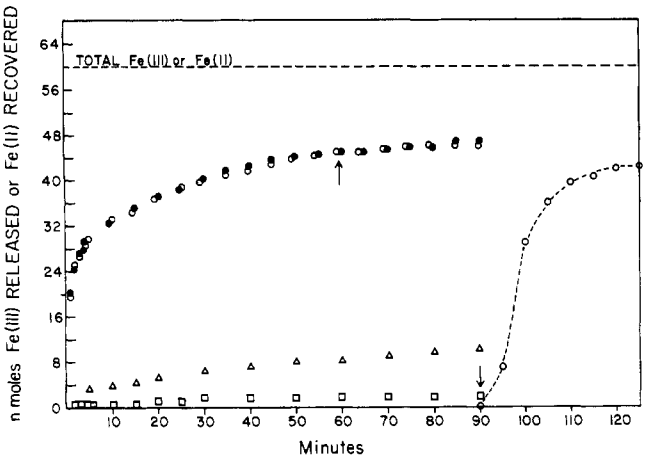


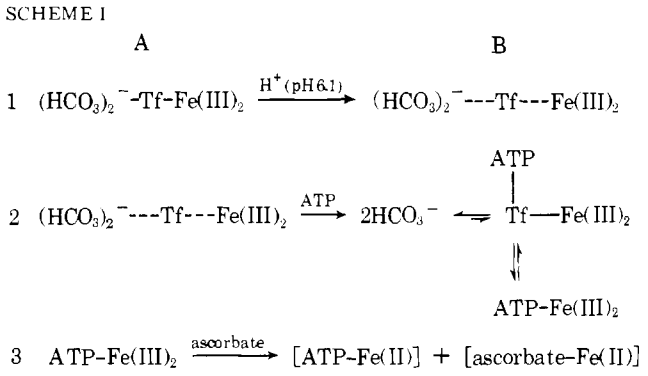
FIGURE 5: The time dependence of Fe(III) release from 30 μM transferrin and the recovery of reduced iron by bipyridine. The reactions were monitored at 465 or 520 nm from 90 to 125 min. The final concentrations of reactants were 30 μM transferrin (0.13 ODU at 465 nm), 0.1 mM ascorbate, 1 mM ATP, and 0.2 mM bipyridine in a total volume of 1 mL, pH 6.1, at 20 °C. The buffer was 25 mM Mes/160 mM NaCl: (□) Tf only; (△) 0.1 mM ascorbate; (●) 1 mM ATP; (○) 1 mM ATP with 0.2 mM bipyridine added at 60 min (indicated by ↑), and 0.1 mM ascorbate added at 90 min (indicated by ↓).

native structure is 2,3-diphosphoglycerate. The observation that AMP and phosphate do not release iron (Figure 3) is in agreement with Egyed's (1975) data for iron and bicarbonate release from transferrin. The loss of iron from transferrin in the presence of a suitable reactant, e.g., ATP, is sensitive to changes in pH, temperature, and NaCl concentration (Figures 1–5). The effects of pH and temperature are in agreement with data presented by Morgan (1977) who suggested that sensitivities to these parameters "represent the link between cellular metabolism and iron uptake".

An increase in ionic strength, the addition of 160 mM NaCl (Figure 1), resulted in an increase of about 1 pH unit at which a measurable rate of iron release was observed. A shift of only 0.2 of a pH unit was obtained in the presence of 1 mM ATP. Since the isoelectric pH of transferrin is 5.6, we may assume from Figure 1 that only a positively charged form of transferrin is capable of releasing iron. Since most proteins show an increased isoelectric pH as ionic strength is increased, we must offer an alternative rationale for the data in Figure 1. One effect of higher salt concentration would be to reduce the water available as one of the Fe(III) coordinating ligands, thereby destabilizing the complex. A less likely explanation is that the high chloride ion concentration competes with apotransferrin for Fe(III).

The chemical scheme below (Scheme I) suggests a possible mechanism for the release of iron from transferrin and the ultimate utilization of iron as Fe(II) for cellular processes, i.e. heme and hemoglobin synthesis. This scheme emphasizes the role of nucleotides in the release of iron from transferrin in immature red blood cells. Line 1 represents the effect of the weak acidic environment on transferrin-iron(III). Column B (line 1) suggests a reduction in binding affinity of the bicarbonate, amino acid side chains (i.e., histidyl and tyrosyl), and water ligands as the result of protonation and/or a change in protein conformation. Iron will load onto transferrin at pH 6.1 with NTA and the anionic site will be replaced with bicarbonate after several days (Aisen et al., 1967). Thus, iron and bicarbonate are probably associated at pH 6.1. A local pH reduction in blood cells, as suggested by Aisen (1973), or on the blood cell membrane could be sufficient for the protonation

SCHEME I



of iron transferrin ligands. Morgan and Baker (1969) have suggested the protonation of transferrin as a first step in the removal of iron from transferrin in the reticulocyte cytosol.

Line 2 suggests that at pH 6.1 ATP will release bicarbonate followed by the release of iron(III) and the formation of a soluble ATP–Fe(III) complex. The loss of bicarbonate has been suggested as a prerequisite for iron release (Aisen and Leibman, 1973; Egyed, 1973; Martinez-Medellin and Schulman, 1973) and can be induced with ATP (Egyed, 1975). The ATP-induced release of bicarbonate is associated with a reduction in absorbancy at 465 nm (Egyed, 1975). In the present study, loss in absorbancy was bimodal for all reactants except reducing agents (Figures 4 and 5), which suggests a preferential loss of iron and/or bicarbonate from one of the two iron-binding sites. Similar bimodal activity for the release of iron and bicarbonate has been observed by other investigators (Aisen et al., 1973; Fletcher and Huehns, 1967; Harris, 1977; Lestas, 1976; Princiotto and Zapolski, 1975).

Experiments (unpublished results) have been performed with 30, 50, 70, and 100% iron-saturated transferrin. In the presence of 1 mM ATP, the rate of iron release was dependent on the concentration of iron and independent of the percent of iron saturation. However, pretreatment of 100% iron-saturated transferrin with 1 mM ATP for 5 min removed more than 30% of the iron. When passed over a G-25 column, the iron remaining on transferrin in the presence of 1 mM ATP was released at a rate which was about one-half that of fully saturated transferrin. Thus, iron appeared to be added to transferrin randomly but selectively released by ATP.

Although ATP may release bicarbonate (line 2), it is not known if iron is simultaneously released from transferrin and chelated with ATP. However, under the weak acidic conditions employed in our study it is possible that ATP formed ATP–iron complexes. The presence of ATP–iron and GTP–iron chelates in red blood cells lends credence to this idea (Bartlett, 1976a,b; Konopka and Szotor, 1972; Konopka et al., 1969). ATP chelates are stable up to pH 8 and have a strong formation constant, i.e. 4×10^6, at pH 2 (Goucher and Taylor, 1964). All trinucleotides tested had the same effect over a narrow pH range (Figure 3b), so the distinguishing factor may be the availability of a trinucleotide. Bartlett (1976a) has shown by competition experiments that GTP has a higher iron binding affinity than ATP. If column B (line 2) is correct, it would appear that ATP may have the capacity to change protein conformation resulting in the release of bicarbonate (Egyed, 1975).

Line 3 represents the interaction of iron with ascorbate and ATP. Based on the data presented, ascorbate has no effect on diferric transferrin but will reduce Fe(III) after the addition of ATP (Figure 5), which supports the idea of the formation of a soluble ATP–Fe complex. The lack of a direct effect by reducing agents, e.g. glutathione, mercaptoethanol, and cys-

teine, on transferrin is supported by Egyed (1973). Fielding and Ryall (1970) suggested that acid dissociation of transferrin ligands was necessary for the action of reducing agents. Existence of an ascorbate–Fe(II) complex was proposed by Zapolski and Princiotto (1977). An ATP–Fe(II) complex may occur, although Bartlett (1976b) was unable to demonstrate its formation when reacting ferrous chloride with ATP or GTP. The continued existence of Fe(II) for potential use by the cell may require a high affinity intracellular chelator to prevent autoxidation.

In summary, we propose a model system of iron release from transferrin involving ATP as an iron-releasing factor. The concentrations of potential iron-releasing molecules used in this study were within physiological levels, with peak activity for ATP below 1 mM. The release of iron was dependent on a weak acidic environment, temperature, and the presence of an iron-releasing agent. At pH 6.1, ascorbate did not release and reduce iron(III) from transferrin efficiently which suggests that iron is still bound to transferrin. Only in the presence of 1 mM ATP was ascorbate able to reduce iron supporting the idea that ATP can release iron in a soluble form, i.e. ATP–Fe(III). The nucleotide–iron complex may then be available for further processing within an immature blood cell either for the formation of iron(III) and iron(II) intermediates or for use directly after reduction to Fe(II) in iron-requiring systems, particularly heme synthesis.

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