An ATP-dependent Iron Transport System in Isolated Rat Liver Nuclei*

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A concerted translational control is responsible for maintaining an iron level in the cytosol that is both adequate for the synthesis of iron-containing proteins and does not represent a danger to the cell. However, little is known about how iron level is controlled in the nucleus. Nuclei of rat liver take up iron from ferric citrate by a process that is dependent on ATP. This system shares several properties with known P-type ATPases, suggesting that a P-type ATPase in the nuclear membrane is responsible for iron transport. (i) Adenosine 5'-[β,γ-iminodiphosphate], a non-hydrolyzable ATP analogue, does not support iron uptake; (ii) the uptake is strongly inhibited by vanadate; (iii) there is an absolute requirement for Mg2+; and (iv) reagents that oxidize SH groups inhibit uptake, and this inhibition can be prevented by dithiothreitol. The energy of activation for the uptake (11.5 kcal/mol) and the Km for ATP (0.4 μm) are similar to values for other known carbon transport ATPases. Inhibitors of Na+,K+-ATPase, sarcoplasmic reticulum Ca2+-ATPase, proton V-ATPase, and nuclear Ca2+-ATPase have no effect on uptake. Ferric citrate can be replaced by Fe-ATP as a source of iron for the transport system; however, two other stronger iron chelators, Tiron and desferrioxamine, completely inhibit the uptake. Taken together, these data strongly suggest that an Fe-ATPase, distinct from other known P-type ATPases, is responsible for iron transport in the nucleus.

Because iron ions are efficient generators of high reactivity oxygen radicals, a balanced cellular control should exist between iron uptake and iron storage in ferritin, a non-reactive iron complex (1). This enables the cell to maintain a pool of free iron adequate for the synthesis of iron-containing proteins but low enough not to cause cell damage. The orchestrated actions that the cells use to achieve this balance in the cytosol have been recently uncovered (2–4); however, nothing is known about the putative control of iron levels in the nucleus.

It is well established that oxidative stress produces DNA damage through the mediation of iron ions present in the organelle (6, 7) and the discovery of both Escherichia coli DNA repair enzymes and transcription factors containing iron-sulfur clusters (8–10).

The iron pool in the nucleus could be a direct consequence of the cytosolic iron control if iron ions would diffuse freely through the nuclear membrane pores. However, a nuclear membrane Ca2+-ATPase, responsible for calcium transport into the nucleus, has recently been found in rat liver (11, 12). This prompted us to investigate the possible existence of a process of ATP-driven iron transport into the nucleus.

EXPERIMENTAL PROCEDURES

Isolation of Nuclei—Nuclei were isolated as described previously (11), except for some minor modifications. Male Wistar albino rats (200–300 g, fed ad libitum) were killed by brain concussion. The liver was cannuled and perfused in situ with 50 ml of deionized ice-cold water to remove blood. The liver was removed, cut into small pieces, and homogenized with a Potter homogenizer (15 strokes) in 40 ml of ice-cold TKM solution (50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM MgCl2). The homogenate was filtered through three layers of cheesecloth, and the nuclei were pelleted by centrifugation at 700 × g for 10 min. The pellets were homogenized (9 strokes) in 40 ml of the TKM solution supplemented with 0.25 mM succrose and centrifuged again at 700 × g for 10 min. The resulting pellet was resuspended in 24 ml of the TKM solution supplemented with 2.3 mM succrose. The tubes were gently mixed, and a 6-ml cushion (TKM containing 2.3 mM succrose) was carefully layered in the bottom of each tube. The tubes were centrifuged at 18,000 × g for 40 min. The upper layer and the sucrose cushion were removed with an aspirator. The resulting pellet (nuclei) was resuspended in TKM containing 0.25 mM succrose and sedimented at 700 × g for 10 min. The pellet of nuclei was resuspended in HKM solution (25 mM Hepes, pH 7.0, 125 mM KCl, 4 mM MgCl2) and centrifuged again at 700 × g for 10 min. The purified nuclei were gently resuspended in HKM. All steps were performed at 0–4 °C. Contamination of the nuclear fraction by plasma membrane fragments was <1%, by microsomes was <2%, and by mitochondria was <2%, as measured by assaying the activities of 5'-nucleotidase, glucose-6-phosphatase, and succinate-cytochrome c reductase, respectively.

Iron Uptake—Isolated nuclei (1800 μg of DNA or 3 × 106 nuclei) were incubated in 1 ml of HKM containing 630 μCi 55Fe-citrate (Amersham Corp., 3 × 10−6 Ci/mCi, 4.7 Ci/mmole) at the indicated temperatures and with the indicated additions. At predetermined times, aliquots of 100 μl were withdrawn, diluted to 1 ml with HKM, and centrifuged at 13,600 × g in an Eppendorf centrifuge (model 5402) for 3 min. The pellet was resuspended in 1 ml of medium and centrifuged again at 13,600 × g for 3 min. The resulting pellet was resuspended in 100 μl of 1% perchloric acid and incubated at 80 °C for 30 min. The resulting suspension was centrifuged at 13,600 × g for 3 min. The supernatant (40 μl) was withdrawn, and the 55Fe radioactivity was determined by liquid scintillation. The molar concentration of 55Fe was estimated from the counts/min values, and the final results were referred to as the amount of nuclear DNA in terms of mol of deoxynucleotides. In experiments in which the uptake rate is plotted, the time course was first carried out for up to 100 min; the uptake rates were then calculated from the total uptake in 60 min, a time that falls within the linear range. In the case of uptake of Fe(III)-ATP, the time course was carried out for up to 20 min.

Protein Concentration—This was determined by the method of Gornall et al. (13) using albumin as a standard.

Materials—All biochemical reagents were from Sigma except 2,5-di-
The complex 55Fe-citrate was made by mixing 10 μM [55Fe(III)]-citrate in 0.1 M HCl with 10 μM ATP. Rat liver nuclei take up iron from consideration to be an important iron carrier in the cell, together used as a source of iron uptake by nuclei because citrate is concentrated, namely, the removal of Fe(III) from citrate, which is a process with a kinetic analysis of iron uptake shows an initial lag followed by a steep increase, which plateaus after 100 min of incubation at a non-inhibitory.

Iron uptake is completely dependent on the presence of Mg2+ (Fig. 4). At 1 mM ATP, the kinetics fits a Michaelis-Menten process with a K_m of 0.5 mM MgCl2. The dependence of iron uptake on ATP concentration is shown in Fig. 5, where it can be seen that optimal conditions are reached at approximately 1 mM ATP. The half-maximal rate was achieved at 0.4 mM ATP. A comparison of the effectiveness of ATP, ADP, AMP, GTP, and AMP-PNP (a non-hydrolyzable analogue of ATP) to support nuclear 55Fe accumulation is shown in Table I. Clearly, ATP provides the strongest stimulus for iron uptake. The low stimulus provided by AMP-PNP suggests the need for ATP hydrolysis. Table I also shows the effects of several specific inhibitors of ATPases on iron uptake. Oligomycin, carbonyl cyanide p-trifluoromethoxyphenylhydrazone, and antimycin A, inhibitors of mitochondrial ATP synthetase (17), showed only a slight inhibitory effect (16%) when added together to the incubation medium. Other ATPase inhibitors were ineffective on the ATP-driven iron uptake. This was the case for 2,5-di-terbutyl-1,4-benzhydroquinoline, a microsomal Ca2+-ATPase inhibitor (11, 18); dicyclohexylcarbodiimide, a specific inhibitor of V- and F-ATPases (17, 19, 20); calmidazolium, a nuclear Ca2+-ATPase inhibitor; ouabain, a specific Na+,K+-ATPase (21, 22); and thapsigargin, a specific inhibitor of sarcoplasmic, endoplasmic reticulum calcium-ATPase (23) and nuclear Ca2+-ATPase (24). Atractyloside, a mitochondrial nucleotide carrier inhibitor (25) and Ap5A, a mioquinase inhibitor (26), were also non-inhibitory.

Also shown in Table I is the partial inhibition of ATP-driven uptake on Fe(III)-citrate concentrations on iron uptake. Isolated nuclei were incubated with the indicated concentrations of 55Fe(III)-citrate for 100 min at 37°C. The uptake rate was measured as indicated under "Experimental Procedures." A, uptake in the presence (filled circles) or absence (open circles) of 1 mM ATP. Data presented are the means ± S.E. of three separate experiments. B, data were plotted to fit the Arrhenius equation. The E_a for iron uptake was 11.3 kcal/mol.

In most of the experiments reported here, Fe(III)-citrate was used as a source of iron uptake by nuclei because citrate is considered to be an important iron carrier in the cell, together with ATP and ADP (14, 15). Rat liver nuclei take up iron from 55Fe(III)-citrate in a process that is dependent on ATP and is affected by temperature (Fig. 1). At 0°C or in the absence of ATP, no iron uptake is observed. At 37°C and in the presence of 1 mM ATP, a strong stimulation of iron uptake occurs. Kinetic analysis of iron uptake shows an initial lag followed by a steep increase, which plateaus after 100 min of incubation at a level corresponding to a ratio of approximately 1 mol of 55Fe to 100 mol of deoxynucleotides. The effect of temperature on iron uptake was investigated in more detail (Fig. 2). In the absence of ATP, there is no visible change in the uptake rate with increasing temperature. However, in the presence of ATP, the dependence is very clear. The Arrhenius plot (Fig. 2B) obtained from the uptake rates in the presence of ATP makes it possible to calculate an E_a of 11.3 kcal/mol.

Fig. 3 shows the dependence of 55Fe uptake on Fe(III)-citrate concentration. In the absence of ATP, there is no change in iron uptake with increasing concentrations of Fe(III)-citrate. In the presence of ATP, the uptake rate increases sharply in the 0–100 μM range and then levels off. The curve does not fit Michaelis-Menten kinetics. This is possibly due to the complexity of the system that may have at least two distinct components, namely, the removal of Fe(III) from citrate, which is a kinetically slow process (16), and the iron transport across the membrane, both of which are dependent on Fe(III) concentration. The maximum 55Fe uptake rate is 140 μmol iron (mol deoxynucleotide)^-1 (min)^-1, which is reached at approximately 150 μM Fe(III)-citrate.

1 The abbreviations used are: E_a, energy of activation; AMP-PNP, adenosine 5′-(β,γ-iminodiphosphate); Ap5A, P1,P3-di(adenosine 5′)-pentaphosphate.
iron uptake (45%) by 100 μM vanadate, an inhibitor of ATP-dependent ion pumps (27). To investigate whether this iron uptake system involves sulfhydryl groups, as in the case for other ATPases (28, 29), N-ethylmaleimide was added to the incubation medium. In this case, 33% inhibition of iron uptake was observed that could be prevented by the simultaneous addition of dithiothreitol. The N-ethylmaleimide concentration employed was 5 mM, which is reported to inhibit other P-ATPases (28, 29). Table I also shows that Tiron and desferrioxamine, two strong Fe(III) chelators, fully inhibited iron uptake.

ATP is also considered to be a cellular iron chelator (15). Therefore, we tested the effectiveness of the complex Fe(III)-ATP in supporting iron uptake by nuclei (Fig. 6). The uptake rate reaches 380 μmol iron (mol deoxynucleotide)−1 (min)−1 at approximately 250 μM Fe(III). The complex Fe(III)-AMP-PNP was much less effective in supporting iron transport across the nuclear membrane, indicating that the process requires ATP hydrolysis. The inclusion of 100 μM vanadate inhibited uptake by over 40% (not shown), similar to that which was observed when Fe(III)-citrate was used as a source of iron. The uptake from Fe(III)-ATP is also completely dependent on Mg2++, and the kinetics of uptake differs from that of Fe(III)-citrate in that there is no initial lag and the plateau is reached at 20 min (not shown).

The effect of up to 2.5-fold concentrations of several metal ions on iron uptake from Fe(III)-citrate is shown in Fig. 7. No uptake inhibition was observed for Ni(II), Pb(II), Mn(II), and Co(II). Slight inhibitions of 20 and 30% were observed for Cu(II) and Al(III), respectively, and they seem to keep inhibitions steady at this level. Catalase was added in these assays because it was possible that auto-oxidation of some of these ions gives rise to superoxide anion and hydrogen peroxide.

### Table I

Effect of nucleotides, ATPase inhibitors, and iron chelators on iron uptake

| Additions | Percentage of activity |
|-----------|------------------------|
| ATP       | 100                    |
| None      | 23                     |
| ADP       | 65                     |
| AMP       | 51                     |
| GTP       | 61                     |
| AMP-PNP   | 40                     |
| ATP + oligomycin + FCCP + antimycin A | 84 |
| ATP + tBuBHQ | 80 |
| ATP + DCCD | 90 |
| ATP + ouabain | 104 |
| ATP + calmidazolium | 90 |
| ATP + thapsigargin | 100 |
| ATP + vanadate | 55 |
| ATP + NEM | 67                     |
| ATP + NEM + DTT | 93 |
| ATP + ATR | 91                     |
| ATP + Ap5A | 99                     |
| ATP + DFO | 2.3                    |
| ATP + Tiron | 2.5                   |

FIG. 4. Effect of increasing Mg2+ concentrations on iron uptake. Isolated nuclei were incubated with the indicated MgCl2 concentrations and 315 μM 55Fe(III)-citrate for 100 min at 37°C. The uptake rate was measured as indicated under “Experimental Procedures.” Data presented are the means ± S.E. of three separate experiments.

FIG. 5. Effect of increasing ATP concentrations on iron uptake. Isolated nuclei were incubated with the indicated ATP concentrations and 315 μM 55Fe(III)-citrate for 100 min at 37°C. The uptake rate was measured as indicated under “Experimental Procedures.” Data presented are the means ± S.E. of three separate experiments.

FIG. 6. Effect of increasing Fe(III)-ATP and Fe(III)-AMP-PNP concentrations on iron uptake. Solutions of 55FeCl3 and ATP (filled circles) or AMP-PNP (open circles) were added to the incubation medium to give the indicated final concentrations of Fe-nucleotide and a 2 mM total concentration of nucleotide (free + iron-bound). Incubation was for 20 min at 37°C, and the uptake rate was measured as indicated under “Experimental Procedures.” Data presented are the means ± S.E. of three separate experiments.

FIG. 7. Effect of increasing Mg2+ concentrations on iron uptake. Isolated nuclei were incubated with the indicated MgCl2 concentrations and 315 μM 55Fe(III)-citrate for 100 min at 37°C. The uptake rate was measured as indicated under “Experimental Procedures.” Data presented are the means ± S.E. of three separate experiments.
We describe here that rat liver nuclei accumulate iron in an ATP-dependent manner when the external source of Fe(III) is provided in the form of Fe(III)-citrate or Fe(III)-ATP, two iron complexes thought to be physiologically important (14, 15). It has been shown that approximately the same amounts of chelatable Fe(II) and Fe(III) are present in rat liver cells (30). In separate experiments (not shown) carried out under the same conditions but using non-radioactive Fe(III)-citrate, the nuclear iron content, determined spectrophotometrically, increased 20-fold in 40 min from 0.29 to 6.0 mmol iron (mol deoxyribonucleotide)$^{-1}$. Evidence strongly suggests that ATP-driven iron accumulation in the nucleus is supported by a P-type ATPase. (i) The uptake does not occur in the absence of ATP. Other nucleotides like ADP, AMP, and GTP support iron accumulation, although with a low efficiency. The partial sustenance of cation transport by nucleotides other than ATP has been reported for other ATPases (11, 24, 31) and in some cases attributed to a loss of specificity due to experimental conditions. (ii) The non-hydrolyzable ATP analogue AMP-PNP was poorly effective in supporting iron uptake, strongly indicating the need for ATP hydrolysis (11, 24, 31) and in some cases attributed to a loss of specificity due to experimental conditions. (iii) The transport is completely dependent on Mg$^{2+}$, indicating the true substrate for the ATPase is MgATP. It is a characteristic of the ATP-driven cation transport cycle of P-type ATPase to use MgATP as a substrate (22, 31, 32). (iv) The uptake is strongly inhibited by 100 μM vanadate, a phosphatase analogue. Vanadate binds to the phosphorylation site of P-type ATPase and appears to lock the enzyme in the $E_2$ state. In the present case, inhibition was not total. This has also been observed in the case of other P-type ATPases (11, 27, 32). One possible explanation is that phosphate in the medium competes with vanadate for the phosphorylation site (27). This might well be the case in our experimental conditions since we have detected a strong ATP hydrolysis by what seems to be an apyrase activity. This has, so far, hampered investigations on the correlation between iron uptake and ATP hydrolysis in this system. (vi) At 5 μM, N-ethylmaleimide inhibited ATP-dependant iron uptake, the inhibition was abolished by dithiothreitol, indicating the involvement of sulfhydryl groups in the process and agreeing with what has been reported for other P-type ATPases involved in cation transport (28, 29).

The $E_a$ for ATP-driven iron uptake was 11.3 kcal/mol. The $E_a$ for Ca$^{2+}$-uptake by vesicles of the sarcoplasmic reticulum has been reported to be 15–17 kcal/mol, while the $E_a$ for ATP hydrolysis in the same system was 14–17 kcal/mol (31). However, if these vesicles were treated with detergents, the $E_a$ increased to 16–27 kcal/mol (31, 33), indicating that the conformation of the enzyme is correlated with $E_a$ values. It is possible that the low $E_a$ value obtained for iron transport is connected with the use of intact nuclei in which the ATPase conformation is close to the native form.

Another parameter of the ATP-dependent iron uptake in nuclei that can be compared with that of other P-type ATPases is the $K_m$ for ATP, which is 0.4 mM. This value compares with those of Na$^+$,K$^+$-ATPase, 0.45 mM (22), Ca$^{2+}$-uptake by rat liver microsomes, 1.8 mM (34), and the second apparent $K_m$ for ATP hydrolysis of sarcoplasmic reticulum Ca$^{2+}$-ATPase, 0.05–0.2 mM (31). A higher value, 4.7 mM, was recently found for a presumptive Cu$^{2+}$-ATPase (32).

The kinetics of iron uptake from Fe(III)-citrate shows an initial lag that we presume to be due to the low rate with which Fe(III) is transferred from the Fe(III)-citrate to the putative iron binding site of the ATPase. Indeed, Fe(III)-citrate is a spherical polymer of molecular weight 2.1 × 10$^6$ from which desferrioxamine and pyridone derivatives remove iron with very slow kinetics (16). The pseudo-first order constant indicates that the limiting step is not dissociation of Fe(III)-citrate complex but rather the formation of the intermediate ternary complex Fe(III)-citrate-ligand (16). If such a complex has to be formed between Fe(III)-citrate and the putative iron binding site of the ATPase, it would explain the initial lag. The same lag was not observed in the iron uptake kinetics from Fe-ATP (not shown), suggesting that iron transfer from ATP to the putative iron binding site of the ATPase is much faster. The complete inhibition of iron uptake by two strong Fe(III) chelators, desferrioxamine and Tiron, indicates the integrity of the nuclei. It also indicates that Fe(III) strongly bound to ligands cannot be transferred to the putative iron binding site of the ATPase.

Recently, iron uptake by endosomal V-ATPase was described (19). It occurs preferentially with Fe(II), is not associated with ATP hydrolysis, and is strongly inhibited by dicyclohexylcarbodiimide. The V-ATPase is part of the system that internalizes iron from Fe-transferrin, and it was considered that Fe(II) uses the proton pore of the V-ATPase to gain access to the cytosol (19). The iron uptake we describe is not affected by dicyclohexylcarbodiimide. It is not affected either by specific inhibitors of other known P-type ATPases and must be ascribed to a new type of P-ATPase.

Intracellular free iron is also in the form of Fe(II) (30). In experiments in which Fe(II) salts or Fe(III)-citrate + ascorbate were used as sources of external Fe(II), some iron uptake was observed, however, that was totally ATP-independent (not shown).

Mutants of E. coli in the iron uptake system (feo) have been isolated (35). Two genes, feoA and feoB, comprise the system. The Feo B protein is localized in the cytoplasmic membrane, and its sequence revealed regions of homology to ATPases, which indicates that iron uptake may be ATP-driven.

Some recent important discoveries in the area of P-type ATPases involved in cation transport refer to a Ca$^{2+}$-ATPase (19) and to a Cu$^{2+}$-ATPase (36, 37). In the experiment in Fig. 7, the inhibitory action of several metal ions on iron uptake by

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2 S. Gurgueira and R. Meneghini, unpublished results.
nuclei was tested, and no significant effect was observed. Thus, it seems that we are dealing with a putative Fe-ATPase, distinct from other P-type ATPases involved in cation transport. It has been shown that iron, in addition to inducing ferritin translation (38), activates ferritin gene transcription in liver (39). Iron may be active in other genes as well. Moreover, the possibility cannot be dismissed that iron plays some structural role in chromatin, as copper does (40). Our recent investigations using electron energy-loss spectroscopic imaging revealed an unexpected concentration of iron in condensed chromatin.2

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