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Iron Binding and Autoreduction By Citrate: Are These Involved in Signalling By Iron Regulatory Protein-1?

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Ferric ions bind to citrate and undergo an autoreduction to form a ferrous-citrate complex, greatly increasing the redox activity of the iron complex. Ferrous ions and citrate are also essential for the enzymic activity of aconitase. Aconitase, with its iron-sulphur cluster has a versatile structure which allows it to act as an iron regulatory protein (IRP-1). The purpose of this study was to see whether iron binding, and its autoreduction by citrate, could play a physiological signalling role in iron regulation. Significant amounts of ferrous ions were associated with citrate, when measured using ferrozine, however, these did not appear to activate iron-requiring aconitase.

Keywords: Iron regulatory protein, iron autoreduction, signalling, iron binding, redox active iron

Abbreviations: IRP-1, iron regulatory protein-1; TfR, transferrin receptor; PIPES, 1,4-piperazinediethanesulphonic acid; LMrFe, low molecular mass iron

Citrate is a biological ligand in mammals for the binding and solubilization of ferric ions.[1] Iron bound to citrate remains redox active and can participate in electron transfer reactions leading to the formation of damaging free radicals, such as the hydroxyl radical.[2] It is also a form of iron that can promote microbial virulence. Recently, it was shown[3] that when ferric ions are complexed to citrate they undergo an autoreduction to yield a ferrous-citrate complex which is considerably more active in decomposing hydrogen peroxide in the Fenton reaction.

Citrate and ferrous ions are also essential for the enzymatic activity of aconitase; an enzyme that catalyses the reversible isomerization of citrate and isocitrate in the citric acid cycle. Aconitase has a versatile structure which allows it to act as an iron regulator of mRNA translation, and is therefore also known as iron regulatory protein-1 (IRP-1) (reviewed in 4). This monomeric protein contains a single iron-sulphur cluster [4Fe-4S] which can reversibly dissociate to the inactive [3Fe-4S] form.[5] When cellular iron levels are low IRP-1 modulates transferrin receptor (TfR) mRNA expression, and when iron is replete ferritin mRNA is expressed. In this way iron uptake and storage can be regulated in a

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co-ordinated manner (reviewed in 6). The purpose of this study was to see whether autoreduction of iron bound to citrate might play a physiological role in iron regulation through activation of iron regulatory protein-1.

MATERIALS AND METHODS

Ferric chloride, ammonium ferrous sulphate, citric acid, ferric citrate, ferrozine, tricarballylic acid, $NADP^+$, and ascorbic acid were obtained in the highest grades from the Sigma Chemical Company, Poole, Dorset, UK. Aconitase was purified from porcine heart, and in the assay one unit (U) of activity was that which catalysed the conversion of 1.0 μmol of citrate to isocitrate per min, pH 7.4, 25°C. Isocitrate dehydrogenase was purified from yeast, and one unit of activity catalysed conversion of 1.0 μmol of isocitrate to oxoglutarate per min, pH 7.4, 37°C. The iron-requiring form of aconitase was prepared as described previously.[7]

Autoreduction of Ferric Citrate

1 mM solutions of ferric chloride and citric acid were prepared in double distilled water. A 1 mM solution of ferric citrate was also prepared. Solutions of iron citrate were incubated at 37°C for up to 3 hours, and longer where indicated. The formation of a ferrous species was detected, and measured against a standard of ammonium ferrous sulphate, using the iron chelator ferrozine, and an absorbance of 562 nm.

Activation of Iron-requiring Aconitase

The following reagents were placed into new, clean, plastic UV microcuvettes:-50 μl of sample or standard; 0.37 ml of PIPES buffer 600 mM, pH 7.4; 0.1 ml sodium citrate, 50 mM; 10 μl sodium azide, 50 mM; 20 μl tricarballylic acid, 100 mM; 20 μl magnesium sulphate, 300 mM; 20 μl $NADP^+$, 20 mM; 10 μl isocitrate dehydrogenase, 50 U/ml; 5 μl aconitase, 126 U/ml. Final volumes were made to 0.8 ml with Chelexed water. The reaction mixture was placed in a recording spectrophotometer and incubated at 37°C. The rate of change at A340 nm was recorded for approximately 45 mins. A dose response calibration using a pure ferrous salt standard was constructed under the same conditions.

RESULTS

As previously observed, solutions of ferric citrate and of ferric salt plus citric acid undergo autoreduction to produce a ferrous citrate complex. After 3 hours, around 25 μmoles/l of ferrous species were formed, whereas after 3 days some 150 μmoles were formed (Figure 1).

At each time point, 50 μl of iron-citrate complex was removed for addition to the iron-requiring aconitase assay to assess its ability to activate the enzyme. As a control, a ferrous salt standard

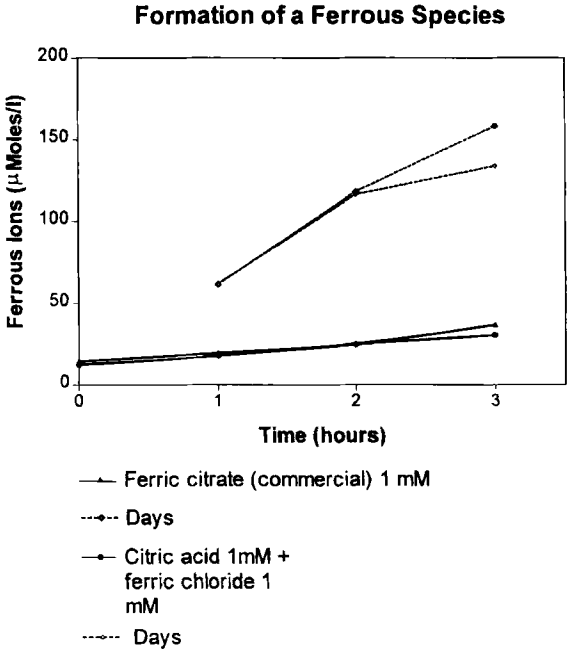

FIGURE 1 Formation of a ferrous citrate complex from ferric citrate as a function of time. Ferrous ions were measured and quantitated using the ferrozine assay.

was included and treated in the same way as the iron-citrate complex (see Fig. 3). By the inclusion of a ferrous salt standard it is also possible to quantitate iron with the aconitase assay.[8] Although some 25 μmoles/l of ferrous species could be detected in ferric citrate incubated for 3 hours, using the ferrozine assay, the same samples showed no reactive iron in the aconitase assay.

Iron-citrate salts incubated for up to 3 days, however, did weakly activate aconitase showing values for ferrous species of between 0.25–1.25 μmoles/l. These values are some 120 to 600 times lower than those detected by the ferrozine assay, depending upon the iron-citrate complex used (Figure 2). When a mixture of ferrous ammonium sulphate and citric acid (1:1), at a concentration of 10 μM, was added to the aconitiase assay it did not stimulate activity whereas the ferrous salt alone (10 μM) did (see Fig. 3).

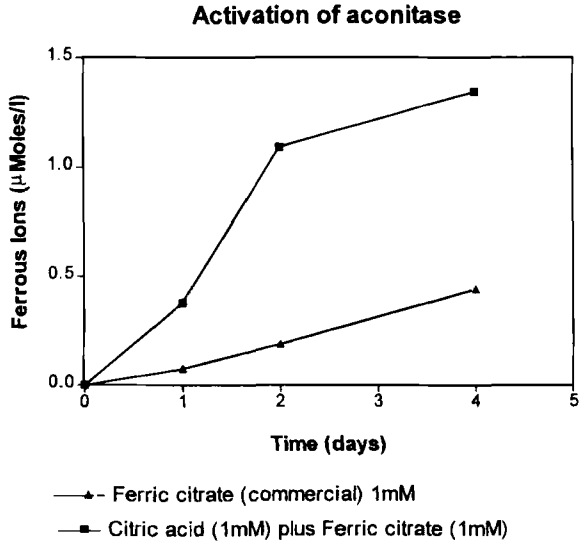

FIGURE 2 Ferrous citrate formed by autoreduction of ferric citrate was tested for its ability to activate iron-requiring aconitase. Reactive iron was quantitated using the aconitase assay.

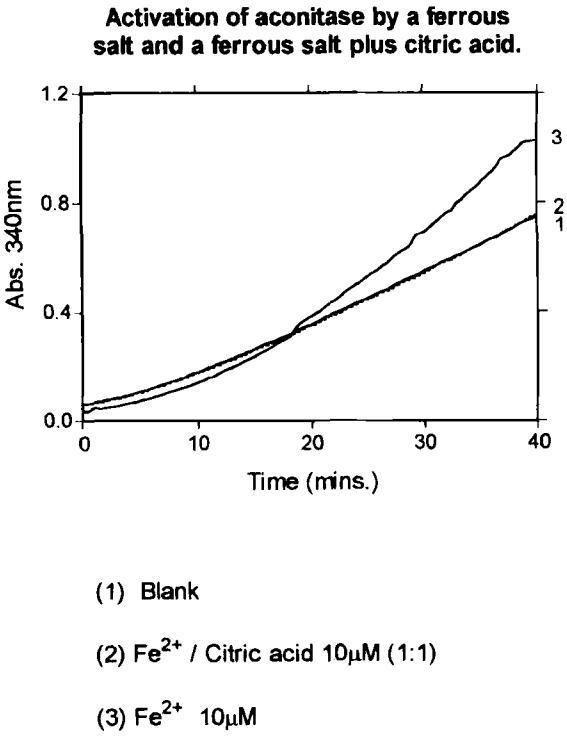

FIGURE 3 Activation of iron-requiring aconitase by a ferrous salt (10 μM) and a mixture of ferrous salt plus citric acid (1:1) 10 μM.

DISCUSSION

The iron-requiring form of aconitase [3Fe-4S] is activated by ferrous irons to form [4Fe-4S] (equation 1).

$$[3Fe-4S]^{1+} + Fe^{2+} = [4Fe-4S]2^{+} \quad (1)$$

This reaction has been exploited to measure total non-haem iron in plasma[7] as well as low molecular mass forms of iron (LMrFe) in plasma,[8,9] likely to participate in redox reactions leading to the formation of oxygen free radicals and other reactive oxygen species. Using the latter reaction, we have sought to establish whether the binding and autoreduction of iron on citrate plays an important role in iron signalling through iron regulatory protein-1. Although significant amounts of ferrous species could be detected bound to citrate, using the ferrozine

assay, most of these were ineffective at activating iron-requiring aconitase. Two possible explanations for this anomaly are that the aconitase binding site for its substrate citrate, may keep citrate-bound ferrous ions away from the iron-sulphur cluster. Alternatively, ferrous ions formed during autoreduction of ferric citrate are too tightly bound to the citrate to be released to activate the iron-sulphur centre [3Fe-4S]. This latter possibility is suggested by the finding that the Fe^{2+} chelator ferrozine, could remove up to 600 times more ferrous iron from citrate, and by the fact that a ferrous salt did activate aconitase whereas a mixture of ferrous salt with citric acid did not.

It, therefore, seems unlikely that citrate iron-binding and subsequent autoreduction are physiological mechanisms for iron signalling unless there are mechanisms operating *in vivo* to direct the iron to the iron-sulphur centre. The data also suggest that the use of ferroin-type chelators, such as ferrozine, to measure biologically reactive LMrFe in biological fluids may substantially over estimate such forms of iron.

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