Iron and Aconitase Activity

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Aconitase activated with Fe^{2+} , cysteine and ascorbate incorporates 1 g-atom of Fe^{2+} /mol. Loss of this Fe^{2+} by transfer to ferrozine, a Fe^{2+} chelator, results in loss of activity. Ascorbate increases the rate of transfer of the essential Fe^{2+} whereas citrate retards the rate of transfer. Transfer of Fe^{2+} from inactive aconitase, 2 g-atoms of Fe /mol, can be accomplished in the presence of urea and ascorbate. The correlation of activity with the presence of an added g-atom of Fe^{2+} /mol leads to the conclusion that active aconitase has only one active site per mol.

The availability of pure aconitase in inactive form (Kennedy *et al.*, 1972) makes possible, in principle, structural comparison of inactive and active aconitase, a comparison that as yet has not been attempted. The present paper is concerned with such a comparison in terms of spectral properties, incorporation of Fe^{2+} on activation, and correlation of loss of added Fe^{2+} with loss of enzyme activity.

To do this active aconitase was prepared, free of activating reagents, by activation of inactive aconitase with Fe^{2+} in the combined presence of ascorbate and cysteine and separation of activating reagents from active aconitase by gel filtration through Sephadex G-25.

Materials and Methods

Aconitase was prepared as previously described (Kennedy *et al.*, 1972) and 0.5 ml portions (2–5 mg of enzyme) in individual tubes were stored at -20°C . NADP^+ , L-ascorbate, 2-(*N*-2-hydroxyethyl)piperazine-*N'*-yl)ethanesulphonic acid (Hepes), isocitrate dehydrogenase (EC 1.1.1.42) and cysteine hydrochloride were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Tricarballic acid was obtained from Eastman Organic Chemicals, Rochester, N.Y., U.S.A., triethanolamine hydrochloride, heavy-metal free, was from Calbiochem, Los Angeles, Calif., U.S.A., and Sephadex gels were from Pharmacia, Piscataway, N.J., U.S.A. Ferrozine, 5,6-diphenyl-3-(2-pyridyl)-1,2,4-triazine-*pp'*-disulphonic acid disodium salt trihydrate, was obtained from Aldrich Chemical Co., Milwaukee, Wis., U.S.A. Ferrous ammonium sulphate hexahydrate, reagent grade, was obtained from Fisher Chemical Corp., Pittsburgh, Pa., U.S.A.

Enzyme assays were carried out at 26°C by the method of Rose & O'Connell (1967) with 0.01 M triethanolamine, pH 8.0, as buffer, 5–10 μl samples being assayed in total volumes of 0.75 ml or 1.50 ml.

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Specific activities are reported in μmol of NADPH formed/min per mg of enzyme protein. Protein concentrations were determined from E_{280} values, by using $E_{1\%}^{1\text{cm}} = 11.5$. Analyses for total iron were carried out by the spectrophotometric method of Henry *et al.* (1958) with *o*-phenanthroline as the reagent, or by the method of Carter (1971) with ferrozine (5.26 mM in water) as the spectrophotometric reagent. For the tris complex of the latter with ferrous iron, $\epsilon = 27900 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ at the absorption maximum, 562 nm (Stookey, 1970, Carter, 1971). Assays and reactions were carried out in 1 cm-light-path cuvettes in the thermostatically controlled cell compartment of a Beckman DU spectrophotometer as modified by Gilford Instrument Laboratories (Oberlin, Ohio, U.S.A.) and with an expanded range recorder. Enzyme activities for a given experimental temperature were monitored at the experimental temperature, activity varying with temperature as follows: 100%, 26°C ; 94.0%, 23°C ; 80.3%, 19°C ; 61.5%, 15°C .

Results

The extent of activation of inactive aconitase is governed by a 1:1:1 equilibrium relationship between the concentration of active sites, ferrous iron and reductant, cysteine (thiol) or ascorbate (Morrison, 1954). The process is time- and temperature-dependent, and to achieve maximal activation of milligram quantities of aconitase within 1 h at room temperature (23°C) the combination of ascorbate and cysteine with ferrous iron has been used. Table 1 presents several results indicating the practicality of the procedure and the necessity of maintaining adequate iron concentration. Essentially, the maximum activation achieved by Rose & O'Connell (1967) with cysteine and ferrous iron is also accomplished with 30 mM-ascorbate, 10 mM-cysteine and 5 mM- Fe^{2+} (activating mixture 2) even at considerable

Table 1. *Activation with ascorbate and cysteine*

Activation and assay were carried out at 23°C with the indicated volume ratios of activating solution to enzyme solution, the latter containing 7.6mg of enzyme/ml. Dilutions before assay were made with 15mM-Tris-tricarballylate, pH7.8. Activating mixture (1) is that used by Rose & O'Connell (1967).

	Activating mixture	Ratio	Time (min)	Dilution	Specific activity
(1)	40mM-Hepes, pH7.4 10mM-Cysteine, pH7.5 2mM-Fe ^{II}	19:1	60	200	9.9
(2)	30mM-Ascorbate, pH7.5 10mM-Cysteine, pH7.5 5mM-Fe ^{II}	4:1	60	200	10.4
		2:1	30	180	10.3
(3)	30mM-Ascorbate, pH7.5 10mM-Cysteine, pH7.5 1.6mM-Fe ^{II}	3:1	30	100	2.5

Fig. 1. Effect of indicated ferrous ion concentration (μM) on the kinetics of activation of aconitase in 30mM-Tris-ascorbate, pH7.5, and 10mM-cysteine, pH7.5, at 20°C

At zero time 50 μl (212 μg) of inactive enzyme was added to 450 μl of activation solution, and 10 μl samples were used for assay. Specific activity at maximum activation was 12.2

dilution of the activating solution with enzyme solution. Additionally, ascorbate effectively prevents air oxidation of cysteine and consequent precipitation of cysteine.

In a typical activation run, 4.24mg of aconitase in 1.0ml of 15mM-Tris-tricarballylate, pH7.8, was activated for 30min at 23°C with 3.0ml of activation solution (30mM-ascorbate, 10mM-cysteine, 5mM-Fe²⁺). Concentration to 1.5ml and gel filtration at 0–2°C, with 15mM-Tris-tricarballylate, pH7.8, on a 1.5cm $\times$ 51cm column of Sephadex G-25 yielded 3.64mg of active aconitase. This represented 86%

Table 2. *Iron content of active enzyme*

In these experiments active enzyme was prepared as described and 1–2mg of enzyme was used for determination of iron, the iron being extracted with hot 5% (w/v) trichloroacetic acid and determined by the method of Henry *et al.* (1958). Inactive enzyme yielded 1.8 g-atoms of iron/mol.

Expt.	Specific activity	Iron content (g-atom/mol)
1	10.0	3.1
2	10.3	2.7
3	8.7	2.7

recovery, specific activity 12.0, in the pool of tubes 15 to 18, 1ml fractions being collected under N₂.

Activation kinetics as a function of Fe²⁺ concentration in the presence of constant concentrations of cysteine and ascorbate show a sigmoidal dependency (Fig. 1) on activation time. A similar sigmoidal dependency has been noted (Villafranca & Mildvan, 1971; Kennedy *et al.*, 1972) in the presence of cysteine and it is apparent that ascorbate has not altered the activation process. The equilibrium nature of activation by Fe²⁺ is indicated by the levelling off of activity at low Fe²⁺ concentrations.

The spectrum of active aconitase is identical in both the u.v. and visible regions with that of inactive aconitase (Kennedy *et al.*, 1972), indicating that both u.v. and visible chromophores of inactive aconitase have not been altered by activation.

Table 2 presents the results of several iron analyses on activated aconitase. The results indicate that 1 g-atom of iron/mol has been incorporated into aconitase on activation. In connexion with the iron-incorporation studies, it is apparent that the reported absorptive capacity of Sephadex for iron (Goldberg, 1973) is not sufficient under the conditions used to remove any substantial amount of iron from activated

Fig. 2. Correlation between iron transfer to ferrozine (E_{562}) and percentage loss of enzyme activity at 26°C

Reaction volumes (800 μ l) contained 458 μ g of active enzyme, specific activity 12.0, 9.4 mM-Tris-tricarballylate, pH 7.8, 12.5 mM-Tris-ascorbate, pH 7.8, and 100 μ l of 5.26 mM-ferrozine added 30 s after the ascorbate. Activities: $\circ$, ascorbate and ferrozine; $\blacksquare$, ascorbate only. E_{562} : $\bullet$, ascorbate and ferrozine; $\blacktriangle$, ferrozine only; 72h values are noted for each curve.

Fig. 3. Time-dependency of ascorbate effect at 20°C

Enzyme added at zero time to ascorbate-ferrozine: $\circ$, E_{562} ; $\bullet$, activity. Enzyme incubated with ascorbate before addition of ferrozine: E_{562} : $\blacksquare$, 30 min; $\blacktriangledown$, 60 min. No ascorbate: $\blacktriangle$, E_{562} . Reaction volumes (400 μ l) contained 109 μ g of active enzyme, 9.4 mM-Tris-tricarballylate, 12.5 mM-Tris-ascorbate, pH 7.8, and 50 μ l of ferrozine reagent. For the enzyme control, $\triangle$, ascorbate and ferrozine were replaced by tricarballylate and water. 72h values of E_{562} are noted for each curve.

enzyme. The possibility that an additional iron atom was added, giving two active sites per mol, and was then removed by gel filtration is ruled out by the fact that specific activity is undiminished and in some instances increases on gel filtration. It may also be noted that incomplete activation leads to decreased iron incorporation and is responsible for a previously reported erroneous result (Kennedy *et al.*, 1972).

The ensuing text and figures present results pertaining to transfer of iron from active aconitase to ferrozine and correlation of iron loss from aconitase with loss of enzyme activity, the results being obtained under a variety of conditions.

Ascorbate markedly enhances the rate of transfer of ferrous iron from active aconitase to ferrozine and causes release of more than one Fe^{2+} ion from active aconitase, the first Fe^{2+} being released at a relatively fast rate (Fig. 2). Extrapolation of the linear segments of the kinetic curve for iron transfer results in an intersect at 1.03 mol of Fe^{2+} removed/mol of active enzyme and, concomitantly, complete loss of enzyme activity is noted (Fig. 2).

Incubation of aconitase with ascorbate for 30 min and 60 min before addition of ferrozine markedly decreases the rate of iron transfer (Fig. 3), although the rate of iron transfer after preincubation remains greater than in the absence of ascorbate. Also, without prior incubation complete transfer of iron (3.06 g-atoms of iron/mol) occurs in 72 h. Controls, not shown in Fig. 3, containing ascorbate showed approx. 25% loss of activity over a 30 min incubation period.

Fig. 4. Effect of citrate and ascorbate at 22°C on iron transfer to ferrozine

Reaction volumes (400 μ l) contained 204 μ g of active enzyme (specific activity 10.1), 9.4 mM-Tris-tricarballylate, 2.5 mM-trisodium citrate or 12.5 mM-Tris-ascorbate, pH 7.8, and 50 μ l of ferrozine reagent, the ferrozine being added immediately after the citrate or ascorbate. Activities: $\square$, citrate; $\blacksquare$, ascorbate. E_{562} : $\blacktriangle$, citrate; $\bullet$, ascorbate; 72h values are noted for each curve.

Fig. 5. Correlation of iron transfer and activity loss as percentages

Iron remaining: ∇ , ascorbate; $\circ$, citrate. Activity remaining: $\blacktriangle$, ascorbate; $\bullet$, citrate. Experimental details were as for Fig. 4, the data of Fig. 4 being used for part (a). For part (b), ferrozine was added 30 min after citrate or ascorbate.

Fig. 6. Iron transfer at 15°C to ferrozine (E_{562}) from inactive enzyme

The reaction mixture (450 μ l) contained 7.8 M-urea, 39 mM-Tris-ascorbate, pH 6.5, 325 μ g of enzyme, and 50 μ l of ferrozine reagent. Ferrozine was absent from the blank.

Citrate exerts an inhibitory effect on iron transfer to ferrozine and a protective effect on enzyme activity over the 30 min period in which one iron atom is transferred to ferrozine in the presence of ascorbate.

Only a small fraction of the iron is transferred in the presence of citrate in the first 30 min and this (Fig. 4) increases only slightly in the ensuing 72 h period. In the same period 3.0 g-atoms of iron/mol of enzyme are transferred in the presence of ascorbate. Although a small amount of transfer occurs in the presence of citrate, no concomitant loss of enzyme activity is noted and it is possible that the 140-fold dilution of the reaction in the course of assay with citrate-containing assay solution, reverses the equilibrium:

Both citrate and ascorbate on incubation with enzyme for 30 min exert a protective action on transfer of iron to ferrozine, the rate and extent of transfer being markedly decreased (Fig. 5b) compared with the same reaction period without incubation (Fig. 5a). Incubation with citrate, although decreasing iron availability to ferrozine, is not accompanied by loss of enzyme activity. Incubation with ascorbate is accompanied by a 30% loss of enzyme activity. On addition of ferrozine enzyme-activity loss and iron transfer occur at a slower rate (Figs. 5b and 5a).

Transfer of iron from inactive aconitase to ferrozine can be accomplished in the presence of a denaturant, 7.8M-urea, in addition to ascorbate (Fig. 6). These conditions enhance the rate of transfer of iron from inactive aconitase, the rate being negligible under the conditions used for demonstrating transfer of the added iron of active aconitase. Although the initial rate of transfer under these conditions is not very rapid, the result at 48h indicates a release of 2.14 g-atoms of iron/mol (66000g) of aconitase.

Discussion

The work of Morrison (1954) with a purified preparation established a 1:1 equilibrium relationship between active sites and Fe^{2+} concentration, and dissociation of Fe^{II} from active enzyme was formulated as:

with

$$K_{\text{Fe}^{II}} = \frac{[E_a][\text{Fe}^{II}]}{[E_a \cdot \text{Fe}^{II}]} \quad (2)$$

where E_a represents active protein, Fe^{II} represents ferrous iron essential for activity, and $K_{\text{Fe}^{II}}$ was determined to be $3.9 \times 10^{-6} \text{M}$. The data, however, were obtained at constant cysteine concentration and the calculated dissociation constant is more correctly formulated as:

$$3.9 \times 10^{-6} = K = \frac{K_{\text{Fe}^{II}}}{K_{\text{Fe}^{II} \cdot \text{Cys}}} \times [\text{Cys}] = \frac{[E_a][\text{Fe}^{II} \cdot \text{Cys}]}{[E_a \cdot \text{Fe}^{II}]} \quad (3)$$

where

$$K_{\text{Fe}^{II} \cdot \text{Cys}} = \frac{[\text{Fe}^{II}][\text{Cys}]}{[\text{Fe}^{II} \cdot \text{Cys}]} \quad (4)$$

$[\text{Cys}]$ represents cysteine concentration, and the cysteine- Fe^{II} complex is formulated for simplicity as a 1:1 complex despite the use of an excess concentration of cysteine (0.01M-cysteine).

At the pH (7.5-7.7) of these studies the data of Tanaka *et al.* (1955) for iron-cysteine complexes, with neither reactant in large excess, indicate the primary species to be a 1:1 complex with a solubility-product constant, K_{sp} , of $2.6 \times 10^{-11} \text{M}^2$. This value may be taken as a minimum value for $K_{\text{Fe}^{II} \cdot \text{Cys}}$, and certainly for higher complexes, $\text{Fe}^{II}(\text{Cys})_2$ and $\text{Fe}^{II}(\text{Cys})_3$, this value would be expected to be several orders of magnitude greater.

Accordingly,

$$K_{\text{Fe}^{II}} \approx 3.9 \times 10^{-6} \times \frac{K_{\text{Fe}^{II} \cdot \text{Cys}}}{[\text{Cys}]} \approx 10^{-14} \text{M} \quad (5)$$

and this approximated minimal value of $K_{\text{Fe}^{II}}$ is sufficiently small to impart distinct stability to active aconitase, thereby permitting, as the results indicate, isolation of active aconitase without loss of the additional and necessary g-atom of ferrous iron.

Transfer of this iron to ferrozine can be accomplished (Figs. 2, 3, 4 and 5) under conditions that do not permit transfer of iron from inactive aconitase, transfer from inactive aconitase (Fig. 6) requiring the presence of urea and a lower pH, 6.5. This evidence clearly differentiates the two iron atoms of inactive aconitase from the essential Fe^{2+} of active aconitase. Further, the visible chromophore of inactive aconitase is not altered by the presence of the third iron atom of active aconitase. It appears, then, that the iron atoms of inactive aconitase have not been modified by activation and may therefore be considered to be non-essential for activity if not for activation. However, their behaviour with respect to transfer to ferrozine has been modified by activation, the two non-essential iron atoms now transferring to ferrozine in the absence of urea. This increased availability of the non-essential iron atoms in active aconitase in view of the absence of any spectral changes is most likely to be due to a protein conformational change incident upon activation.

The transfer experiments utilizing ferrozine as a reagent for both trapping and determining ferrous iron serve also to correlate loss of Fe^{2+} from active enzyme with loss of enzyme activity. The results (Fig. 2) demonstrate complete loss of enzyme activity after 1 atom of iron/mol of active enzyme has been transferred to ferrozine. This result is corroborated by the results presented in Figs. 3, 4 and 5, and these figures as well as Fig. 2 also demonstrate the correlation of activity loss with iron transfer over the reaction period necessary for complete enzyme loss. Over this period the correlation of activity loss with iron transfer is reasonable in view of the activity lost, 20-30%, from the controls. This point is particularly well borne out by the data of Fig. 2, the percentage of activity lost exceeding the percentage of iron transfer towards the end of the 30min reaction period, a period during which the control lost 30% activity.

The addition, by activation, of an additional g-atom of iron/mol of aconitase, the correlation of activity with the presence of the additional g-atom of iron and the 1:1 relationship between active sites and Fe^{2+} concentration lead, of course, to the conclusion that aconitase has only one active site per molecule. This conclusion and the fact that citrate markedly inhibits transfer of Fe^{2+} to ferrozine (Figs. 5a and 5b) support present concepts of the enzyme mechanism (Glusker, 1968; Hendrickson & Srere, 1968; Villafranca & Mildvan, 1972), a ternary complex formulated as below being central to the mechanism.

Formulation of the ternary complex in this manner also suggests the possibility of inhibition of enzyme activity by formation of similar ternary complexes with inhibitors providing ligands to Fe^{II} .

$\text{E}_a \cdot \text{Fe}^{II} \cdot \text{Inhibitor}$

With this possibility in mind, the results do not reveal any evidence for ferrozine exerting its effect in this manner but rather show that formation of the tris complex, Fe^{II} (ferrozine)₃, with absorption maximum at 562nm (Stookey, 1970), is responsible for loss of enzyme activity. This is in keeping with the lack of similarity, in both size and structure, between ferrozine and aconitase substrates and with the stability of the tris complex (Stookey, 1970), the formation constant, although not determined (Stookey, 1970), presumably being of the same order (10^{21}) as that for *o*-phenanthroline (Lee *et al.*, 1948).

On the other hand, the results, particularly those presented in Figs. 5(a) and 5(b), point to the possibility of ascorbate exerting an inhibitory effect owing to the formation of a ternary complex

$\text{E}_a \cdot \text{Fe}^{II} \cdot \text{Ascorbate}$

the formation of this complex being time-dependent. Formation of such a complex would decrease both enzyme activity and transfer of Fe^{2+} to ferrozine. The results of Figs. 5(a) and 5(b) support this possibility, incubation of active enzyme with ascorbate for 30min resulting in 33% loss of activity and decreased rate of transfer of Fe^{2+} to ferrozine.

The nature of the activating process remains obscure, the requirement for cysteine (or ascorbate) in a mol-to-mol ratio with enzyme (Morrison, 1954) possibly reflecting the situation found with papain (Gawron & Cheslock, 1951; Smith & Kimmel, 1960; Neumann *et al.*, 1967) and the sigmoidal kinetics found with Fe^{2+} suggesting (Villafranca & Mildvan, 1971) a consecutive reaction of two Fe^{2+} with inactive enzyme to give one active site. On these grounds, it is attractively simple to consider the activation reaction as requiring transfer of electrons

from two Fe^{2+} via the two iron atoms already present in inactive aconitase. In this connexion, the Villafranca & Mildvan (1971) inactive aconitase preparation, reported to contain 1 g-atom of iron/89000g, and manifesting sigmoidal activation kinetics with Fe^{2+} , is of interest. However, we have been unable to obtain aconitase of high specific activity by the Villafranca & Mildvan (1971) method so that comparison is not possible.

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