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## Kinetic Studies on the Two-stage Activation of Aconitase

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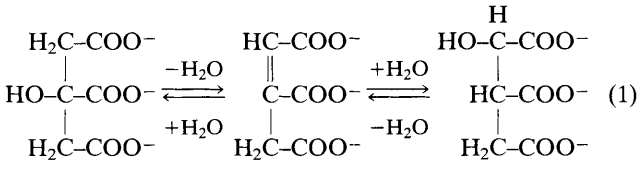
Kinetic studies on the stepwise activation of aconitase $[3Fe–4S]^{+} \rightarrow [3Fe–4S]^{0}$ (reduction), followed by $[3Fe–4S]^{0} \rightarrow [4Fe–4S]^{2+}$ ($Fe^{2+}$ incorporation) are described.

The enzyme aconitase catalyses the dehydration–rehydration process resulting in the interconversion of citrate and *iso*-citrate *via* the allylic intermediate *cis*-aconitate [eqn. (1)], a reaction which is a part of the Kreb's cycle.[1]



(1)

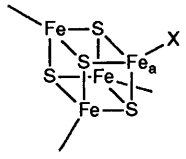
In this work aconitase was isolated from fresh beef hearts using a modified form of existing procedures.[2–5] The purity of the protein was estimated to be >95% by comparing its activity with literature values.[5] The crystal structure of pig heart aconitase has been refined to 2.5 Å resolution.[6] Active aconitase has a $[4Fe–4S]^{2+}$ cuboidal cluster close to the centre of the molecule ($M_r \sim 80{,}000$; 755 amino acids), which is accessed by a cleft.[7] One of the Fe's in the cluster designated $Fe_a$, coordinated by X (either $H_2O$ or $OH^-$) is labile. In the course of the aerobic isolation $Fe_a$ is lost and the inactive $[3Fe–4S]^{+}$ containing enzyme is obtained.[5] *In vitro* activation can be achieved by reducing $[3Fe–4S]^{+}$ to $[3Fe–4S]^{0}$ with dithionite, followed by $Fe^{2+}$ incorporation to give $[4Fe–4S]^{2+}$ [eqns. (2) and (3)].

$$[3Fe–4S]^{+} + \tfrac{1}{2}S_2O_4^{2-} + H_2O \rightarrow [3Fe–4S]^{0} + SO_3^{2-} + 2H^{+} \quad (2)$$

$$[3Fe–4S]^{0} + Fe^{2+} \rightarrow [4Fe–4S]^{2+} \quad (3)$$

The different states of aconitase are shown in Scheme 1. All the forms indicated except $[4Fe–4S]^{3+}$ have been identified. Approximate reduction potentials for the $[3Fe–4S]^{+}/[3Fe–4S]^{0}$ (−0.16 V) and $[4Fe–4S]^{2+}/[4Fe–4S]^{+}$ (−0.50 V) couples have been indicated.

Here we report for the first time kinetic studies relating to the activation process, including the trimer → cube conversion. Relevant spectra are indicated in Fig. 1.[8] Samples of inactive $[3Fe–4S]^{+}$ enzyme were made air-free by (Amicon) diafiltration using air-free solutions. These and dithionite solutions ~0.01 M (1 M = 1 mol $dm^{-3}$) were made up in a Miller-Howe glove box ($O_2 < 5$ ppm). A 20-fold excess of edta over enzyme was used to complex any free $Fe^{2+}$. In kinetic runs the variation of [edta] showed no effect on $k_{obs}$ provided $[Fe^{2+}]$ is corrected for the amount of edta (3–14%) added. Solutions were made up at pH 7.4 using 85 mM HEPES buffer (Sigma Chemicals), and the ionic strength $I$ was adjusted to 0.100 M with NaCl. Solutions of iron(II) ammonium sulphate (BDH, Analar), or iron(II) perchlorate (G. F. Smith, USA) were used as the source of $Fe^{2+}$. Aconitase concentrations were in the range $(1.5–2.5) \times 10^{5}$ M.




Both reactions were studied by stopped-flow spectrophotometry. All solutions were transferred manually from the glove-box to the stopped-flow by syringe.

For the dithionite reduction of inactive $[3Fe–4S]^{+}$ aconitase the reaction was monitored at 440 nm. The rate law conforms to the empirical eqn. (4).

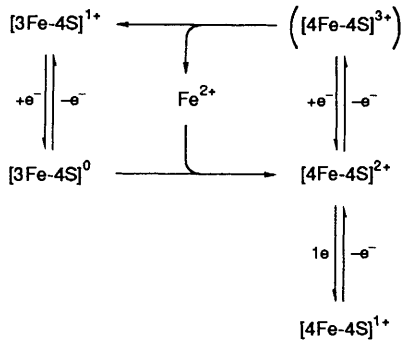
$$k_{obs} = \frac{[S_2O_4^{3-}]^{\frac{1}{2}}}{a[S_2O_4^{2-}]^{\frac{1}{2}} + b} \quad (4)$$

Thus a plot of $[k_{obs}]^{-1}$ *vs.* $[S_2O_4^{2-}]^{-\frac{1}{2}}$ is linear, range of $[S_2O_4^{2-}]$ employed (0.16–8.55) $\times 10^{-3}$ M. The behaviour observed is consistent with the mechanism shown in eqns. (5)–(7), where (5) and (6) are rapid. The equilibrium constant $K_d$ has been determined previously and is $1.4 \times 10^{-9}$ M.[9] From the present study $K = (2.62 \pm 0.03) \times 10^{6}$ $M^{-1}$ and $k = 0.44 \pm 0.01$ $s^{-1}$ at 25 °C.

$$S_2O_4^{2-} \overset{k_d}{\rightleftharpoons} 2SO_2^{\bullet -} \quad (5)$$

$$[3Fe–4S]^{+} + SO_2^{\bullet -} \overset{K}{\rightleftharpoons} [3Fe–4S]^{+}, SO_2^{\bullet -} \quad (6)$$

$$[3Fe–4S]^{+}, SO_2^{\bullet -} \xrightarrow{k/H_2O} [3Fe–4S]^{0} + SO_3^{2-} + 2H^{+} \quad (7)$$



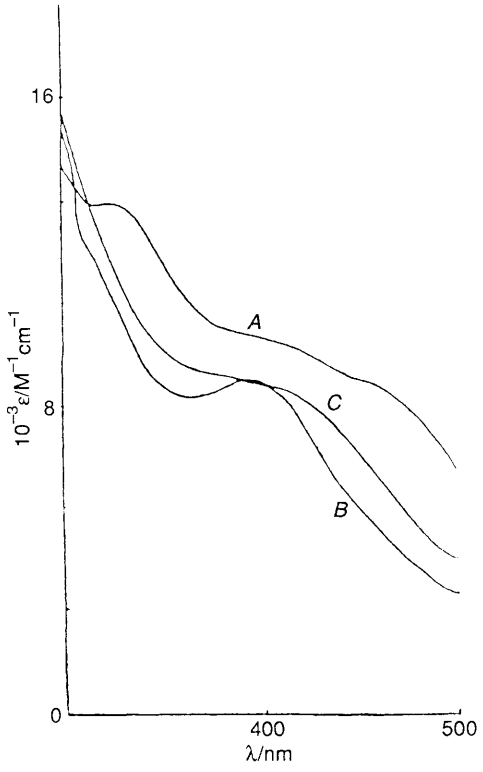



**Fig. 1** UV–VIS spectra of aconitase clusters, $[3Fe\text{-}4S]^{+}$ in 90 mM HEPES (pH 7.5) (*A*); $[3Fe\text{–}4S]^{0}$ obtained on reduction of *A* (*B*); and $[4Fe\text{–}4S]^{2+}$ after $Fe^{2+}$ incorporation in 90 mM (pH 7.5) (*C*).[8] [HEPES = *N'*-(2-hydroxyethyl)piperazine-*N*-ethanesulphonic acid.]

In order to study the reaction with $Fe^{2+}$, the $S_2O_4^{2-}$ reduction was first carried out in the glove-box. Solutions were then transferred to the stopped-flow, and the formation of $[4Fe\text{–}4S]^{2+}$ monitored at 440 nm. The rate law with no sulphate present is as in eqn. (8).

$$\text{Rate} = k_{obs}\,[3Fe\text{–}4S^{0}] \quad (8)$$

where

$$k_{obs} = k_o + k_1[Fe^{2+}] \quad (9)$$

With sulphate present the $FeSO_4$ complex also participates, see eqn. (10).

$$k_{obs} = k_o + k_1[Fe^{2+}] + k_2[FeSO_4] \quad (10)$$

Under the condition $[SO_4^{2-}] >> [Fe^{2+}]$ the following dependence see eqn. (11) has been shown to apply, where $K$ is for the formation of $FeSO_4$ from $Fe^{2+}$ and $SO_4^{2-}$.

$$\frac{(k_{obs}-k_o)(1+K[SO_4^{2-}])}{[Fe^{2+}]} = k_1 + k_2K[SO_4^{2-}] \quad (11)$$

Using a literature value $K = 2.17\ M^{-1}$,† rate constants (25 °C) are $k_1 = 1.99 \pm 0.11\ M^{-1}\ s^{-1}$ and $k_2 = 269 \pm 12\ M^{-1}\ s^{-1}$, indicating a much enhanced reaction of neutral $FeSO_4$ over $Fe^{2+}$. The presence of charged residues in the cleft is noted. One reason for investigating the effect of $SO_4^{2-}$ relates to the observation that in the crystal structure sulphate is present within the molecule and close to the active site. The function and/or significance of the $SO_4^{2-}$ is not clear. The term $k_o = (2.83 \pm 0.53) \times 10^{-3}\ s^{-1}$ involves a prior association of $Fe^{2+}$ with enzyme. Since $[4Fe\text{–}4S]^{2+}$ is the product from this step, one possibility is that it arises from inactive $[3Fe\text{–}4S]^{0}$ which has already become associated with $Fe^{2+}$.

Investigations are at present being extended to include reactions of other 2+ transition-metal ions with $[3Fe\text{–}4S]^{0}$, which become incorporated at the $Fe_a$ site. The chemistry of such mixed-metal clusters and nature of the Fe specificity are of interest.

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† The formation constant $K$ for $FeSO_4$ was estimated from consideration of the following equilibria,

$$Fe^{2+} + SO_4^{2+} \overset{k_{12}}{\rightleftharpoons} Fe^{2+}, SO_4^{2-} \overset{k_{23}}{\rightleftharpoons} FeSO_4$$

where $K = K_{12}K_{23} = [FeSO_4]/[Fe^{2+}][SO_4^{2-}]$. Values of $K_{12} = 13\ M^{-1}$ and $K_{23} = 0.167$ were from refs. 10,11.