Kinetic studies on the dithionite reduction of beef-heart aconitase in the [3Fe–4S]$^+$ inactive form

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Abstract

The kinetics of the dithionite reduction of the [3Fe–4S]$^+$ form of aconitase has been studied at 25 °C in 85 mM HEPES, pH 7.4, $I=0.100$ M (NaCl). The half-order dependence on $[S_2O_4^{2-}]$ indicates that the $SO_2^{\cdot-}$ radical is the effective reducing agent. Saturation kinetic behaviour is observed which can be rationalised in terms of a mechanism involving association of $SO_2^{\cdot-}$ and enzyme ($K=2.64\times10^6$ M^{-1}) prior to reduction of the [3Fe–4S]$^+$ cluster ($k=0.44$ s^{-1}). Alternative mechanisms are considered. No reduction of the [3Fe–4S]$^+$ cluster is observed with other sulfur-containing reducing agents including dithiothreitol (DTT), SO_3^{2-}, $S_2O_3^{2-}$ and L-cysteine. Implications of these various findings are considered.

Introduction

Aconitase [citrate-isocitrate hydrolyase EC 4.2.1.3], the second enzyme in the Krebs cycle, catalyses the interconversion of citrate and isocitrate via the allylic intermediate *cis*-aconitate. Though, aconitase was identified by Martius in 1937 [1], it was found to contain an Fe/S active site only as recently as 1972 [2]. The enzyme is unusual in that the Fe–S cluster catalyses an isomerisation process and is not involved in electron transfer. Present knowledge on aconitase is largely attributable to the work of Beinert and co-workers during the last decade [3–5].

Crystal structures of both the active and inactive forms of pig heart aconitase have been published [6, 7]. According to these structures the catalytically active form of aconitase contains a single [4Fe–4S]$^{2+}$ cuboidal cluster in a molecule of 755 amino acids (M_r 80 000). The cluster is located in a pocket close to the centre of the molecule [6]. The inactive enzyme contains a [3Fe–4S]$^+$ centre which has an incomplete cuboidal geometry. The Fe/S cluster in aconitase is attached to the protein via three Fe–SR bonds, where RS$^-$ represents a cysteinate residue. The fourth Fe atom of the active form, generally designated as Fe_a, is attached to H_2O or OH, Fig. 1. When aerobically isolated, aconitase is catalytically inactive. The [3Fe–4S]$^+$ cluster of the inactive enzyme can be activated in two distinct chemical steps, reduction of the [3Fe–4S]$^+$ cluster

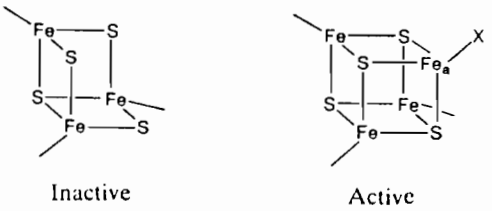

Fig. 1. The Fe–S clusters of inactive and active forms of aconitase, X = H_2O, OH, Fe_a of the active form is lost upon oxidative inactivation of the enzyme.

followed by incorporation of Fe^{2+}, eqns. (1) and (2) [8, 9].

$$[3Fe\text{–}4S]^+ + \text{Reductant} \longrightarrow [3Fe\text{–}4S]^0 \quad (1)$$

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

Interestingly, it is also possible to activate the enzyme with a reducing agent only [8]. In such cases the Fe is supplied by decomposition of some of the existing incomplete Fe–S clusters.

Though reduction of the [3F–4S]$^+$ cluster is a prerequisite for activation of the enzyme, little is known about the process itself. In this paper we describe the kinetic behaviour of the reduction step using $S_2O_4^{2-}$ as reducing agent. The study was carried out at physiological pH 7.4 (85 mM HEPES buffer), constant ionic strength, $I=0.100$ M (NaCl), and temperature 25 °C. Reduction of the enzyme with other sulfur containing reagents cysteine, SO_3^{2-}, $S_2O_3^{2-}$ and dithiothreitol (DTT), was also explored. The work has been referred to in a communication on aconitase studies [10].

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Experimental

Protein

Aconitase was isolated from fresh beef hearts using a modified form of existing procedures [8, 11–13]. Air-free solutions of aconitase were obtained either by dialysis or by Amicon diafiltration using air-free buffer solutions, 85 mM HEPES, pH 7.4. Reagent solutions were also made up in air-free HEPES buffer in a Miller–Howe glove box with $O_2 < 5$ ppm. Ionic strength of both the protein and reagent solutions were adjusted to 0.100 M with NaCl. For each run a fresh solution of $S_2O_4^{2-}$ was prepared and standardised spectrophotometrically against $[Fe(CN)_6]^{3-}$, $\epsilon = 1010\ M^{-1}\ cm^{-1}$ at 420 nm [14]. In order to avoid incorporation of any free Fe^{2+} into $[3Fe\text{–}4S]^0$, a 20-fold excess of the disodium dihydrogen salt of *N,N,N′,N′*-ethylenediaminetetraacetate (edta) over the enzyme was added to enzyme solutions.

Other reagents

The compounds *N*-2[hydroxyethyl]piprazine-*N*-[2-ethanesulfonic acid] (HEPES; Sigma), L-cysteine hydrochloride (Sigma), sodium dithionite ($Na_2S_2O_4$; Fluka), DL-dithiothreitol (DTT; Sigma), sodium sulfite (Na_2SO_3; BDH) and sodium thiosulfate ($Na_2S_2O_3$; BDH), NaCl and $NaClO_4$ (both Analar BDH) were used as supplied.

Physical measurements

UV–Vis spectra were recorded on a Perkin-Elmer Lambda 9 UV–VIS spectrometer. Reaction kinetics were monitored on a Dionex D-110 stopped-flow spectrophotometer by following the decay of the protein absorbance at 440 nm, Fig. 2. Data were collected and analysed on an IBM PC/AT-X computer using software from On-Line Instrument Systems (OLIS), Jefferson, CA, USA. All experiments were carried out with $[S_2O_4^{2-}] > 10 \times$ [protein]. The protein concentration was in the range $(1.5\text{–}2.5) \times 10^{-5}$ M. A detailed stopped-flow kinetic study of the reaction of aconitase with dithionite was carried out. However no reaction was observed over periods of ~1 h with SO_3^{2-}, $S_2O_3^{2-}$, cysteine and DTT as reductant, with concentration of aconitase $< 50 \times$ [Reductant]. Studies were at 25 °C, pH 7.4 (85 mM HEPES buffer) and $I = 0.100$ M (NaCl) under anaerobic conditions.

Results

First-order rate constant k_{obs} for the $S_2O_4^{2-}$ reduction of the aconitase active site $[3Fe\text{–}4S]^+$ to $[3Fe\text{–}4S]^0$ are listed in Table 1. The variation observed in Fig. 3(a) corresponds to saturation kinetics. Thus a plot of $[k_{obs}]^{-1}$ versus $[S_2O_4^{2-}]^{-1/2}$, Fig. 3(b), gives a linear plot with a positive intercept. The rate law is therefore of the empirical form, eqn. (3)

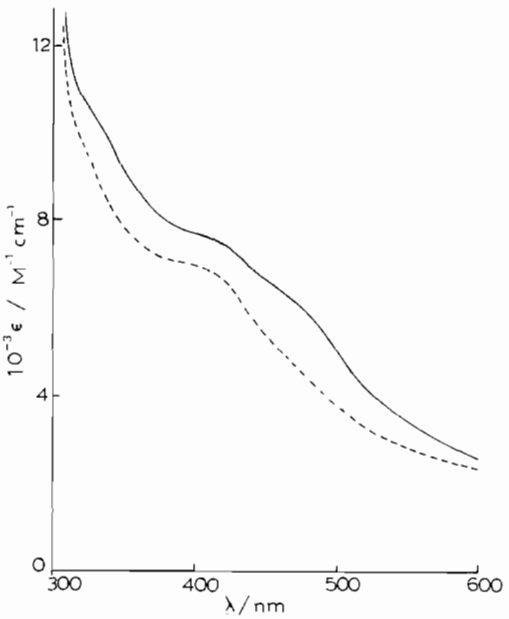

Fig. 2. UV–Vis spectra of $[3Fe\text{–}4S]^{1+}$ aconitase (top trace) and $[3Fe\text{–}4S]^0$ aconitase (bottom trace) in 85 mM HEPES, pH 7.4, $I = 0.1$ M (NaCl).

$$\frac{1}{k_{obs}} = a + \frac{b}{[S_2O_4^{2-}]^{1/2}} \tag{3}$$

From Fig. 3, $a = 2.25(\pm 0.02)\ s^{-1}$ and $b = 2.30(\pm 0.03) \times 10^{-2}\ M^{1/2}\ s^{-1}$. Upon rearrangement, eqn. (3) gives eqn. (4).

$$k_{obs} = \frac{[S_2O_4^{2-}]^{1/2}}{b + a[S_2O_4^{2-}]^{1/2}} \tag{4}$$

The dependence of k_{obs} on $[S_2O_4^{2-}]^{1/2}$ indicates that the radical $SO_2^{\bullet-}$ rather than dimeric $S_2O_4^{2-}$, is the reducing agent. The mechanism (5)–(7) is consistent with these observations.

$$S_2O_4^{2-} \overset{K_d}{\rightleftharpoons} 2SO_2^{\bullet-} \tag{5}$$

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

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

If the prior equilibria (5) and (6) are established rapidly then the last stage of reduction (7) is rate determining. No effects on k_{obs} were observed on replacing Cl^- by ClO_4^-, adjusting I with SO_4^{2-} instead of Cl^-, and doubling the amount of edta (Table 1). Equations (6) and (7) yield the rate-law dependence (8).

$$k_{obs} = \frac{Kk[SO_2^{\bullet-}]}{1 + K[SO_2^{\bullet-}]} \tag{8}$$

TABLE 1. First-order rate constant (25 °C) for the reaction of $[3Fe\text{–}4S]^+$ aconitase, $(1.5\text{–}2.0)\times10^{-5}$ M, with dithionite the reactant in large excess, pH 7.4 (85 mM HEPES), $I=0.100$ M

$10^3[S_2O_4^{2-}]$ (M)	0.16	0.21	0.26	0.32	0.38	0.40	0.45	0.61	
k_{obs} (s^{-1})	0.226	0.264	0.274	0.274	0.287	0.297	0.302	0.311[a,b]	
$10^3[S_2O_4^{2-}]$ (M)	0.78	0.83	1.00	1.57	1.65	2.75	3.62	6.39	8.55
k_{obs} (s^{-1})	0.324	0.307[c]	0.338[d]	0.351	0.357[c]	0.373	0.382	0.397	0.400

[a]On replacing Cl^- by ClO_4^-, $k_{obs}=0.317$ s^{-1}. [b]With excess of edta 40-fold not 20-fold, $k_{obs}=0.320$ s^{-1}. [c]Ionic strength adjusted with SO_4^{2-} instead of Cl^-. [d]On replacing Cl^- by ClO_4^-, $k_{obs}=0.322$ s^{-1}.

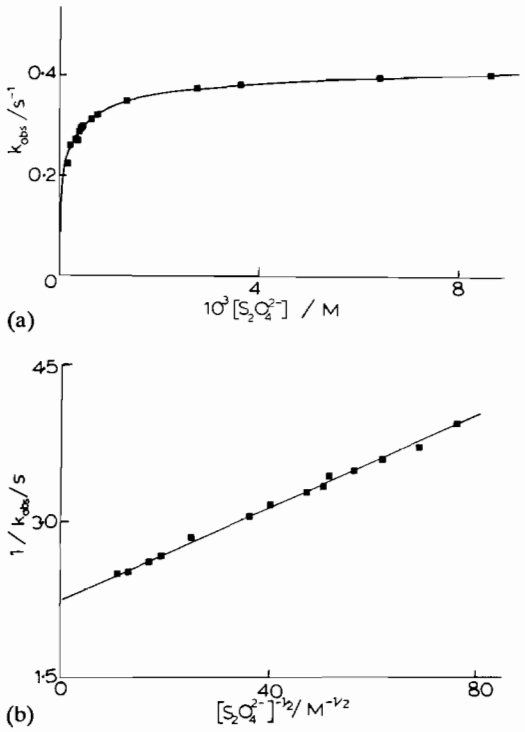

Fig. 3. Plot of (a) k_{obs} against $[S_2O_4^{2-}]$ and (b) $1/k_{obs}$ against $1/[S_2O_4^{2-}]^{1/2}$ for the reaction of $[3Fe\text{–}4S]^+$ aconitase with $S_2O_4^{2-}$ at 25 °C, pH 7.4 (85 mM HEPES), $I=0.100$ M.

Since $[SO_2^{\bullet-}]=K_d^{1/2}[S_2O_4^{2-}]^{1/2}$ from eqn. (5), eqn. (9) can be derived

$$k_{obs}=\frac{KkK_d^{1/2}[S_2O_4^{2-}]^{1/2}}{1+KK_d^{1/2}[S_2O_4^{2-}]^{1/2}} \quad (9)$$

which is similar to the empirical eqn. (4). Using the literature value of $K_d=1.4\times10^{-9}$ M [15], values of the equilibrium constant K and the rate constant k are $(2.62\pm0.03)\times10^6$ M^{-1} and 0.44 ± 0.01 s^{-1}, respectively.

Discussion

Dithionite is a strong two-electron reductant, eqn. (10), which can react in a single two electron or two

$$S_2O_4^{2-}+2H_2O \rightleftharpoons 2HSO_3^-+2H^++2e^- \quad (10)$$

successive one-electron steps. Reduction potentials (versus NHE) are -0.66 and -0.18 V, for the $SO_2^{\bullet-}/HSO_3^-$ and $S_2O_4^{2-}/HSO_3^-$ couples at pH 7.0, respectively [16, 17]. The rapid reduction of the $[3Fe\text{–}4S]^+$ cluster of aconitase solely by $SO_2^{\bullet-}$ requires some discussion. With the reported value of $E^0=-0.16$ V for the $[3Fe\text{–}4S]^+/[3Fe\text{–}4S]^0$ couple of aconitase [18], and the relatively high concentration of $S_2O_4^{2-}$ over $SO_2^{\bullet-}$, K_d for eqn. (5) is 1.4×10^{-9} M [15], one might have expected a significant contribution from the reaction with $S_2O_4^{2-}$ as reductant. Two possible reasons can be suggested. The first is that the reduction potential of the $[3Fe\text{–}4S]^+/[3Fe\text{–}4S]^0$ couple may be more negative than the reported value of -0.16 V, which is only a preliminary value. Furthermore, we were unable to reduce the enzyme with DTT ($E^0=-0.332$V) [19a], $S_2O_3^{2-}$ ($E^0=0.08$ V for the $S_4O_6^{2-}/S_2O_3^{2-}$ couple) [19b], and cysteine ($E^0=-0.21$ or -0.33 for the cystine/cysteine couple) [20, 21]. The enzyme can be reduced by cysteine and DTT in the presence of Fe^{2+}, which suggests that the Fe^{2+} complexes of these reagents are sufficiently strong reducing agents for reaction to occur. The second related reason is the higher chemical reactivity of $SO_2^{\bullet-}$ in terms of E^0. We note that molecules such as citrate and sulfate (see crystal structure [6, 7]) can access the active site of aconitase, and $S_2O_4^{2-}$, although bigger and having a higher charge density than $SO_2^{\bullet-}$, is unlikely to be excluded on these grounds. The half-order dependence on $[S_2O_4^{2-}]$ supporting the involvement of $SO_2^{\bullet-}$ is unequivocal.

Kinetic studies on dithionite reductions of a number of other redox active metalloproteins as well as inorganic molecules have been reported [15, 17, 22, 23]. In one case at least both $SO_2^{\bullet-}$ and $S_2O_4^{2-}$ were found to be reactive [15]. Interestingly, in the majority of studies, the $SO_2^{\bullet-}$ radical is the effective reducing agent. Ferredoxin (spinach) [15], nitrite reductase (*Wolinella succinogenes*) [23], lumiflavin-3-acetate [15], metmyoglobin (horse heart) [17] and a number of its adducts MbX [22] (X = H_2O, OH, imidazole F^-, N_3^-, CNO^-, SCN, HCO_2^-, NO_2^-, CN^-), as well as dioxygen [15, 24] and hydrogen peroxide [24], give rate dependencies for the reaction with dithionite (in large excess) of the form $k_{obs}=a[S_2O_4^{2-}]^{1/2}$. In all cases therefore this supports

a mechanism involving $SO_2^{\bullet -}$ as the kinetically important reducing species.

In the light of these reports, and taking the various reduction potentials at their reported values, it is not surprising that $SO_2^{\bullet -}$ reduces $[3Fe–4S]^+$ to $[3Fe–4S]^0$. However, the way in which it accomplishes this, by saturation kinetics, is unexpected. Since replacement of Cl^- by ClO_4^- (~ 0.06 M), and adjustment of the ionic strength by SO_4^{2-} instead of Cl^- has no effect (Table 1), it is unlikely that Cl^- is implicated in the mechanism. Also important is the observation that increasing the level of edta from 20 to 40-fold has no effect on k_{obs}. Questions relate to the lifetime of $SO_2^{\bullet -}$ and of the adduct [3Fe–4S], $SO_2^{\bullet -}$. An alternative would be to invoke association of $S_2O_4^{2-}$ with aconitase, since a 2−reactant is more likely to interact in this way with a positively charged region on aconitase. Dissociation of $S_2O_4^{2-}$ within the adduct might then be a possibility. The two equilibria (5)–(6) thus become interchanged. However this scheme by introducing eqn. (11) will affect the rate law, which will be of a less simple

$$[3Fe–4S]^+, S_2O_4^{2-} \rightleftharpoons [3Fe–4S]^+, SO_2^{\bullet -} + SO_2^{\bullet -} \quad (11)$$

form. A further proposal that $SO_2^{\bullet -}$ reduces a site X (not specified) on the protein in an equilibrium process could also explain the saturation kinetic behaviour. For a large enzyme molecule such as aconitase, it is difficult to comment further on this possibility. It should also be mentioned that saturation behaviour has been observed for the dithionite reductions of the metmyoglobin adducts Mb^+X ($X = N_3^-$, CNO^-, NO_2^-, HCO_2^-) [22] and nitrite reductase [23, 25]. In the case of Mb^+X, the behaviour is rationalised in terms of the equilibrium $Mb^+X \rightleftharpoons Mb^+ + X$ which is equivalent to eqn. (6), while in the case of nitrite reductase a redox equilibrium has been proposed.

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