Rate Law for the Linearisation of the Incomplete Cuboidal $Fe_3S_4^+$ Active Site in Aconitase at High pH†

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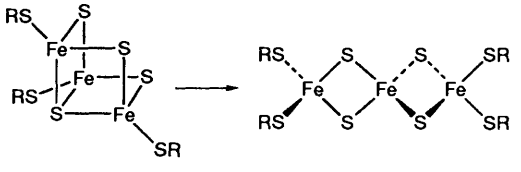
The conversion of the inactive form of aconitase, with an incomplete cuboidal (Fe_a metal depleted) $Fe_3S_4^+$ active site, to the linearised $Fe(\mu\text{-}S)_2Fe(\mu\text{-}S)_2Fe^+$ form at high pH (9.5–10.6) has been studied kinetically. A uniphasic process requiring up to 4 h, with an $[H^+]^{-2}$ rate-law dependence was indicated. Care is required in controlling the pH with a protein of such high molecular weight. In this work buffer concentrations of at least 30 mM were required to avoid pH variations during runs. Even at these concentrations initial pH changes of up to 0.08 were observed on mixing the protein with buffer. Rate constants were found to be independent of amounts of 3-cyclohexylaminopropane-1-sulfonic acid, glycine or the more ionic carbonate buffers. It was not possible to monitor the reverse process involving re-formation of the incomplete cube in the pH range studied.

Aconitase (754 amino acids, $M_r \approx 83\,000$) is a [4Fe–4S] enzyme [citrate (isocitrate) hydrolyase, E.C. 4.2.1.3] which catalyses the isomerisation of citrate to isocitrate *via* the *cis*-aconitate intermediate in the tricarboxylic acid cycle in heart mitochondria.[1] The enzyme from beef heart mitochondria has been extensively characterised by spectroscopic methods including EPR,[2] Mössbauer,[3] and electron nuclear double resonance (ENDOR) techniques.[4] The [4Fe–4S] active site has a labile Fe, and aconitase was one of the first proteins in which the incomplete cuboidal [3Fe–4S] cluster in this case inactive $Fe_3S_4^+$ was shown to be present.[5,6] Robins and Stout[7] have reported the crystal structure for pig heart protein to 2.1 Å resolution. The first structure on crystals grown aerobically from ammonium sulfate solutions in the presence of tricarballylate(propane-1,2,3-tricarboxylate), a weakly binding inhibitor, revealed the presence of the $Fe_3S_4^+$ cluster, a peptide bound sulfate, but no inhibitor. The [4Fe–4S] cluster was obtained by soaking the crystals in iron(II)-dithionite solution, and the structure (to 2.5 Å resolution) showed that a fourth Fe atom, Fe_a, had been taken up by the active site.[8] Crystallographic information on the binding of substrates and inhibitors to this active $Fe_4S_4^{2+}$ enzymatic state has been obtained, and it has been demonstrated that Fe_a binds the substrate (or inhibitor) in a bidentate fashion together with H_2O (or OH^-) making the Fe six-co-ordinate.[9] Properties of aconitase have been reviewed.[10,11]

It has not so far been possible to synthesise small molecular analogues of the $Fe_3S_4^+$ incomplete cuboidal cluster, and all such attempts have resulted in the formation of the linearised $Fe(\mu\text{-}S)_2Fe(\mu\text{-}S)_2Fe^+$ form.[12,13] At high pH (>9.5) aconitase undergoes a brown to purple colour change which has been monitored by UV/VIS spectrophotometry.[13] Low-temperature magnetic circular dichroism (MCD) spectra and MCD properties of the linear $Fe_3S_4^+$ cluster present in solutions of $[NEt_4]_3[Fe_3S_4(SEt)_4]$, and the purple form of beef-heart aconitase have indicated a close equivalence of the two structures.[15] In order better to understand the ability of aconitase to accommodate a linear as well as incomplete cuboidal cluster, we decided to study mechanistic aspects of this reaction. It has been demonstrated that the monodentate cysteine (Cys) residues which bind to the linear cluster are those at positions 250, 257, 421 and 424 in the polypeptide chain, whereas in the incomplete cube the Cys residues are at 358, 421 and 424, as indicated in equation (1).[14]



(1)

Although a multi-stage process is occurring, requiring cleavage of two Fe–S and one Fe–S(Cys), and the formation of one Fe–S and two Fe–S(Cys) bonds, the interconversion conforms to a simple rate law at pH values in the range 9.4–10.6 investigated.

Experimental

Aconitase.—Fresh beef hearts were minced with a kitchenware blender within 3 h of procurement from the beast, and stored frozen at −20 °C until required. Aconitase was prepared from ≈1.5 kg of beef heart mince by a procedure similar to that described elsewhere,[16] and based on earlier procedures.[6,17–19] Typically 120 mg of pure aconitase were obtained.[20] The UV/VIS spectrum of aconitase in 85 mM hepes [*N*′-(2-hydroxyethyl)piperazine-*N*-ethanesulfonic acid] (pH 7.4) confirmed the presence of the $Fe_3S_4^+$ inactive form of the enzyme.[17] The protein was concentrated to about 1.5×10^{-3} M in 2 mM hepes buffer pH 7.4 by ultradialysis, and could be stored air-free indefinitely in ≈25 μl aliquots (from a 200 μl Pipetman) at −80 °C as long as freezing/melting was avoided. Concentrations of the enzyme were determined spectrophotometrically at 280 nm ($\varepsilon = 92\,000$ M^{-1} cm^{-1}).[19]

Buffers.—The following buffers were used: hepes (pK_a 7.5, effective range pH 6.8–8.2), 3-cyclohexylaminopropane-1-sulfonic acid (caps, pK_a 10.4, pH 9.7–11.1), both from Sigma Chemicals, glycine–NaOH (pK_a 9.78, pH 8.6–10.6), sodium hydrogencarbonate–NaOH (pK_a 10.33, pH 9.7–10.9), both from BDH (Analar) and 2-(*N*-cyclohexylamino)ethanesulfonic

† *Non-SI unit employed:* M = mol dm^{-3}.

acid (ches; pK_a 9.3, pH 8.6–10.0) from Sigma. In all cases the pH was adjusted to the value required (Radiometer PHM 62 meter), using a Russell combined Ag–AgCl reference glass electrode (CWR/322), with addition of BDH Convol NaOH solutions of appropriate concentration.

Kinetic Studies.—To commence runs an aliquot of stock $Fe_3S_4^+$ aconitase (typically 20 μl) was added to 1 cm^3 of solution buffered at the required pH, in an optical cell. Mixing was achieved by gently and repeatedly (×4) upturning the Teflon stoppered cell. Initial and final spectra 300–700 nm, Fig. 1, for a run at pH 10.3 indicated 580 nm as the most favourable wavelength to monitor the linearisation process. Reactions were studied under aerobic conditions at 25.0 ± 0.1 °C by conventional ($t_{\frac{1}{2}}$ > 1 min) time range spectrophotometry on a Shimadzu UV 2101 PC instrument which gave absorbance (A) changes with time. Reactions required periods of 10 min to 4 h to proceed to completion. First-order rate constants k_{obs} were obtained from the slopes of plots of $\ln(A_\infty - A_t)$ against time. For the pH range studied A_∞ is not dependent on pH and the reaction proceeds to completion. Ionic strengths were adjusted to 0.100 M with in most cases NaCl (BDH, Analar) or $NaClO_4$ (Fluka, recrystallised twice) or Na_2SO_4 (BDH, Analar) as required. Care was required in adjusting pH and in particular with the amounts of buffer used. Concentrations of buffer were varied from 0.050 to 0.150 M with caps (pH 10.26), 0.035 to 0.050 M with glycine (pH 10.25) and 0.030 to 0.045 M with carbonate (pH 10.21 ± 0.01), without any effects on pH. The carbonate variations were limited by the ionic strength restriction. There were however changes in pH of up to 0.08 within 1 min of mixing the protein stock in 2 mM hepes at pH 7.4 with buffered solutions to initiate linearisation. A number of control experiments were required to define these changes. No further changes in pH were observed for the duration of runs. Final pH values are listed. Most runs were in 0.050 M caps at pH 9.5–10.6.

Results

The effect of pH increases above 9.5 on aconitase $Fe_3S_4^+$ protein were monitored spectrophotometrically. Absorbance increases at 580 nm gave first-order plots linear to three half-lives, from which rate constants k_{obs}, Table 1, were obtained. Particularly important is the observation that the reaction is uniphasic. No effect on rate constants was observed on varying the concentration of caps buffer 0.050–0.150 M, and the latter was therefore fixed at 0.050 M, Fig. 2. The replacement of NaCl by $NaClO_4$ or Na_2SO_4 to adjust the ionic strength had no effect, Table 1. Nor were rate constants affected by using glycine and carbonate buffers, the concentrations of which were also varied, Fig. 2. At pH > 10.6 aconitase undergoes denaturation, and a bleaching effect was observed in the visible range.

The variation of k_{obs} with pH, Fig. 2, suggests a dependence of the kind $[H^+]^{-2}$. A full dependence as in equation (2) can be derived, by considering the mass balance of

$$k_{obs} = kK_1K_2/(K_1K_2 + K_1[H^+] + [H^+]^2) \quad (2)$$

total protein $[Ac]_T = [H_nAc] + [H_{n-1}Ac] + [H_{n-2}Ac]$ in the reaction sequence (3)–(5). Here H_n represents the degree

$$H_nAc \overset{K_1}{\rightleftharpoons} H_{n-1} + H^+ \quad (3)$$

$$H_{n-1}Ac \overset{K_2}{\rightleftharpoons} H_{n-2}Ac + H^+ \quad (4)$$

$$H_{n-2}Ac \xrightarrow{k} \text{linearised product} \quad (5)$$

Table 1 The variation of first-order rate constants k_{obs} (25 °C) for the reaction of inactive $Fe_3S_4^+$ aconitase to the linearised form with pH in 0.050 M caps buffer, I = 0.100 M (NaCl), except as indicated

pH	$10^3k_{obs}/s^{-1}$	pH	$10^3k_{obs}/s^{-1}$
9.55	0.22	10.26	5.1
9.85	0.78	10.27	5.4
10.04	1.6	10.29	5.1
10.06	1.9	10.30	5.3[j]
10.13	3.8	10.34	7.1
10.16	3.5	10.38	8.9
10.20	3.3[a]	10.41	11.6
10.21	4.3[b]	10.43	11.2
10.21	3.3[c]	10.44	13.1
10.22	4.9[d]	10.46	12.9[j]
10.24	4.4[e]	10.47	14.4
10.25	4.6[f]	10.48	15.8
10.26	4.5[g]	10.49	14.4
10.26	5.0[h]	10.53	17.4
10.26	4.6[i]		

[a] Buffer 0.30 M $NaHCO_3$. [b] Buffer 0.035 M $NaHCO_3$. [c] Buffer 0.040 M $NaHCO_3$. [d] Buffer 0.045 M $NaHCO_3$. [e] Buffer 0.035 M glycine. [f] Buffer 0.050 M glycine. [g] Buffer 0.100 M caps. [h] Buffer 0.150 M caps. [i] I = 0.100 M (Na_2SO_4). [j] I = 0.100 M ($NaClO_4$).

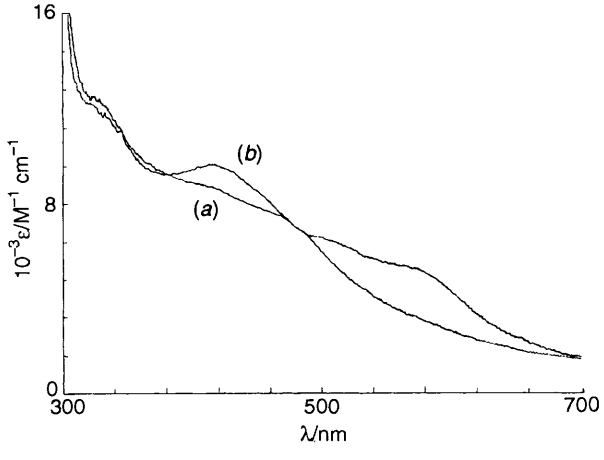


Fig. 1 Spectra of $Fe_3S_4^+$ aconitase in the cuboidal form (a) at pH < 9.5 (lower spectrum at 420 and 580 nm), and in the linearised form (b) at pH 9.5–10.6, I = 0.100 M (NaCl)

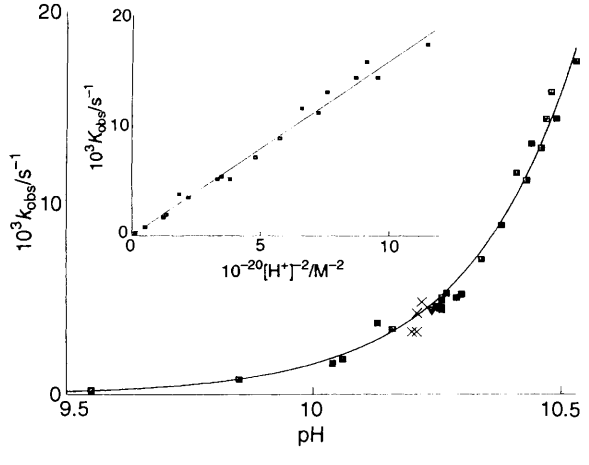


Fig. 2 Plot of k_{obs} (25 °C) *vs.* pH for the linearisation of $Fe_3S_4^+$ aconitase. The effect of different buffers caps (■), Glycine (▼) and carbonate (×), I = 0.100 M (NaCl). Runs in which I was adjusted with $NaClO_4$ and Na_2SO_4 are also included. The inset shows a plot of k_{obs} against $[H^+]^{-2}$, same data

of protonation of aconitase (Ac), with $H_{n-2}Ac$ the reactive form. Equation (2) can be rearranged to give (6).

$$\frac{1}{k_{obs}} = \frac{1}{k} + \frac{[H^+]}{kK_2} + \frac{[H^+]^2}{kK_1K_2} \quad (6)$$

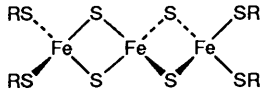
The best linear least-squares fit was obtained for an abbreviated form as in (7), which can be tested for by plotting

$$\frac{1}{k_{obs}[H^+]} = \frac{1}{kK_2} + \frac{[H^+]}{kK_1K_2} \quad (7)$$

$(k_{obs}[H^+])^{-1}$ against $[H^+]$, Fig. 3. The intercept of the latter at $(3.5 \pm 5.8) \times 10^{11}$ M^{-1} s is however poorly defined, with the slope $(5.7 \pm 0.7) \times 10^{22}$ M^{-2} s the most significant feature, consistent with the inset to Fig. 2. The value for K_1 obtained of 6.1×10^{-12} M carries a large error.

Discussion

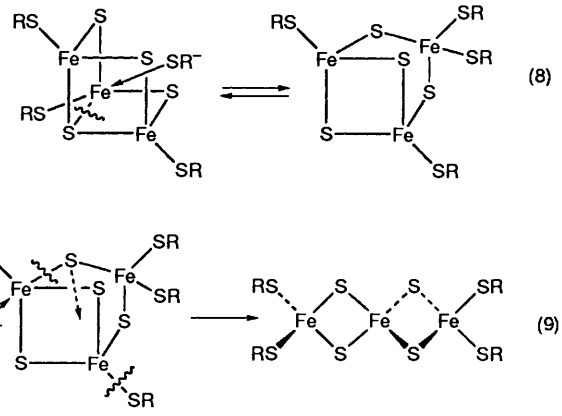
Using radio-labelling techniques Beinert and co-workers[21] have demonstrated that the linearised $Fe_3S_4^+$ form obtained by treating aconitase with 6 M guanidine at pH 8 is attached to cysteine residues 250, 257, 421 and 424. It has not been established to which Fe the new cysteine ligands 250 and 257 are attached. We assume the same linear structure **I** is formed



I

on adjustment of the pH in the present studies, since the UV/VIS spectrum is very similar to that in 6 M guanidine. On examination of the structure of inactive $Fe_3S_4^+$ aconitase by molecular graphics, both cysteines are seen to be far away from the cluster, with Cys-250 (≈17.3 Å) and Cys-257 (≈14.0 Å). There must therefore be significant movements to accommodate the changes under discussion.[22]

Since the reaction is uniphasic with an essentially $[H^+]^{-2}$ dependence, rapid equilibria as in (3) and (4), prior to the rate-determining step (5) can be defined. The Fe attached initially to Cys-358 is the most likely candidate to become the middle Fe in the linearised structure. The need for two deprotonation steps resulting in an $[H^+]^{-2}$ dependence coincides with the observation that two new cysteine ligands Cys-250 and Cys-257 become co-ordinated to the $Fe_3S_4^+$ cluster. Relevant steps involving also two core Fe–S bond cleavages can be written as in equations (8) and (9).



Here it is suggested that the linearisation process is initiated by deprotonation of the cysteines. However there is no evidence for rate-law contributions of the kind $(K_a + [H^+])^{-1}$ involving an acid dissociation process K_a, and the pK_a values appear to be unusually high (certainly > 10.6). Consistent with this an approximate K_1 value of 11.2 is observed from Fig. 3, equation (7). Other residues may of course be implicated, for example arginine residues of which Arg-447 (7.1 Å) and Arg-452 (4.9 Å) are in close proximity to the active site. Alternatively one anomalously high cysteine pK_a (high due to hydrogen bonding) may be involved with a second acid dissociation arising in some other way not here defined. There are a number of ways of implicating the various bond cleavage and bond formation processes.

The linearisation process observed is made possible by the Fe–S cluster of aconitase existing in a pocket which is big enough to accommodate the citrate/isocitrate and *cis*-aconitate moieties involved in the catalytic cycle. The observation that 6 M guanidine (and 8 M urea) also initiate linearisation is presumably more a direct result of unfolding of the aconitase polypeptide. The instability which we observe at pH > 10.6 may be a related unfolding process.

No linearisation process was observed with the dithionite reduced $Fe_3S_4^0$ form at pH 10.35 in 0.050 M caps over a period of > 30 min. The linearisation process therefore appears specific to the higher oxidation state $Fe_3S_4^+$. Also attempts to quantify the conversion of the linearisation active site back to the cuboidal form by decreasing the pH were unsuccessful. Thus the linearised protein is readily denatured on adjusting the pH to ≤8.5 in 0.050 M ches or 0.050 M hepes buffer. The linearised form is stable at pH 9.0 in 0.05 M ches buffer, but when Fe^{2+} (6×10^{-4} M) is added under air-free conditions to induce re-equilibration a white flocculence was again observed. Kennedy *et al.*[14] have reported that the conversion to cuboidal trinuclear form is almost quantitative when the linear form is reduced in the presence of Fe^{2+}. In our experience the reverse reaction is difficult to control and is very sensitive to the conditions employed.

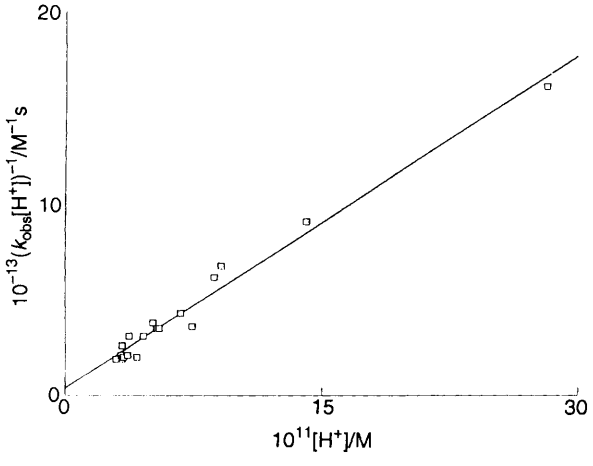


Fig. 3 Plot of $(k_{obs}[H^+])^{-1}$ against $[H^+]$ to test the $[H^+]$ dependence of aconitase $Fe_3S_4^+$ linearisation, equation (7), at 25 °C, I = 0.100 M (NaCl)

Acknowledgements

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