Kinetic Studies on the Reaction of M^{2+} Ions with Aconitase $Fe_3S_4^0$ To Give $Fe_3MS_4^{2+}$ Clusters (M = Fe, Mn, Co)

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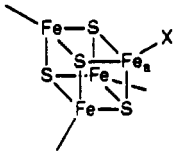
Kinetic studies (25 °C) on the transformation $Fe_3S_4^0 + M^{2+} \rightleftarrows Fe_3MS_4^{2+}$, in which M^{2+} enters the vacant subsite of an [3Fe-4S] incomplete cuboidal structure of aconitase to give $Fe_3MS_4^{2+}$ products, are reported. The studies described with M = Fe are part of the enzyme reactivation process, and the product has been confirmed as the $Fe_4S_4^{2+}$ cluster. With all three metal ions M = Fe, Mn, and Co, uniphasic equilibration processes are observed, yielding at pH 7.4 formation rate constants $k_1/M^{-1}\,s^{-1}$ of 1.9 (Fe^{2+}), 1.0 (Mn^{2+}), and 5.9 (Co^{2+}). Together with rate constants (k_2) for the reverse processes, equilibrium constants $10^{-2}\,K/M^{-1}$ for incorporation of 6.1 (Fe^{2+}), 2.2 (Mn^{2+}), and 13.5 (Co^{2+}) are obtained. In the case of $[Co^{2+}] > 1.5 \times 10^{-3}$ M, a second phase corresponding to a denaturation process has to be allowed for, and with Ni^{2+}, the latter becomes the dominant process, preventing any determination of rate constants. Values of k_1 are $>10^5$ times smaller than rate constants for H_2O exchange on M^{2+} hexaaqua ions, reflecting in part the inaccessibility of the $Fe_3S_4^0$ cluster. Aconitase is a basic protein (pI ~8.5), and the overall electrostatics for reaction with M^{2+} are also unfavorable. More favorable is the reaction with $Fe(H_2O)_5SO_4$ formed on addition of SO_4^{2-}, which reacts 140 times faster than hexaaqua Fe^{2+}. The K values obtained together with those from electrochemical studies on Fe^{2+}, Zn^{2+}, and Cd^{2+} incorporation into the *Desulfovibrio africanus* $Fe_3S_4^0$ cluster are in the order $Cd^{2+} > Zn^{2+} > Co^{2+} > Fe^{2+} > Mn^{2+}$. This order is as expected for the coordination of 2+ metal ions to electron-rich sulfido groups.

Introduction

Aconitase [aconitate hydratase; citrate (isocitrate) hydrolyase; EC 4.2.1.3] in its active form contains a [4Fe-4S] cluster, which catalyzes the nonredox interconversion of citrate and isocitrate molecule via *cis*-aconitate.[1,2] Unlike the [4Fe-4S] clusters in the ferredoxins, which are implicated in biological electron transfer, the cluster in aconitase is at the center of the molecule (M_r ~83 000; 754 amino acids) and is accessed via a channel, i.e. cleft, in the polypeptide matrix. One of the Fe's in the cluster, designated Fe_a and coordinated by X (either H_2O or OH^-), is



labile. Thus in the course of the aerobic isolation Fe_a is lost, and the inactive $Fe_3S_4^+$-containing enzyme is obtained, where $Fe_3S_4^+$ has an incomplete (metal depleted) cuboidal geometry.[1-4] In the presence of a reducing agent and Fe^{2+}, the $Fe_3S_4^+$ cluster is converted into the active $Fe_4S_4^{2+}$ enzymic form.[5] The reactivation process proceeds stepwise with reduction of the $Fe_3S_4^+$ cluster to $Fe_3S_4^0$, followed by incorporation of Fe^{2+} to yield $Fe_4S_4^{2+}$, (1) and (2),[5,6] The kinetics of the reduction of the $Fe_3S_4^+$ cluster of

$$Fe_3S_4^+ + e^- \rightarrow Fe_3S_4^0 \quad (1)$$

$$Fe_3S_4^0 + Fe^{2+} \rightarrow Fe_4S_4^{2+} \quad (2)$$

aconitase by dithionite to give $Fe_3S_4^0$ have been studied.[7] The $SO_2^{\bullet-}$ radical rather than the $S_2O_4^{2-}$ anion has been found to be the effective reducing agent. Although the reactivation of aconitase can be achieved using only a reducing agent, the ultimate need of Fe^{2+} (from another cluster if not provided in the free state) has been proved unambiguously.[5]

The involvement of heterometallic Fe–S-containing clusters in biology is now well established in the case of the Fe–Mo protein of nitrogenase (Fe_7MoS_8 cluster),[8] as well as in the Fe–V-containing nitrogenase,[9] and the existence of other heterometallic clusters seems likely. It is now established that [3Fe–4S] clusters in certain proteins[10] undergo reversible reactions in which a fourth metal atom M^{2+} is incorporated at the vacant site to give a cuboidal product, (3).[11] Such interconversions have been reported previ-

$$Fe_3S_4^0 + M^{2+} \rightleftarrows Fe_3MS_4^{2+} \quad (3)$$

ously for the ferredoxin from *Desulfovibrio gigas* (M = Fe, Co, Zn), and the cuboidal products have been characterized by EPR and Mössbauer spectroscopy.[12-15] There have also been similar reports on the reaction of ferredoxin III from *Desulfovibrio*

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africanus (M = Fe, Zn, Cd, and Tl as Tl^+), where the product has been characterized by EPR, MCD, and electrochemical studies,[16,17] and for the ferredoxin from *Pyrococcus furiosus* (M = Zn, Ni).[18,19] However the possibility of Fe_3NiS_4 participation in the carbon monoxide dehydrogenase of *Rhodospirillium rubrum* was recently disproved, the Ni being separated from and in close proximity to rather than being a part of the cluster.[20] Procedures for the preparation of analogue complexes containing Fe_3MS_4 clusters (M = Fe, Co, Ni, V, Mo, W, Re) are well documented.[21-31]

This paper is concerned with the kinetics of aconitase reactivation involving as a prime process addition of hexaaqua Fe^{2+} to $Fe_3S_4^0$. Reactions were studied in noncomplexing perchlorate and sulfate-containing aqueous solutions at pH 7.4 (85 mM Hepes), $I = 0.100$ M. Because of the increasing interest in and relevance of heterometallic clusters, we have also studied the incorporation of other metal ions, including hexaaqua Mn^{2+} and Co^{2+}, in perchlorate and report equilibrium constants for all three processes, (3). The results obtained provide for the first time kinetic information on metal incorporation into Fe_3S_4 clusters.

Experimental Section

Protein. Aconitase was isolated from beef hearts by the following procedure.[5,32-37] Fresh beef hearts were used in all cases. All procedures were carried out in a cold room at 0–4 °C unless otherwise indicated. The hearts were cleaned from fats and connecting tissues and minced through a coarse domestic mincer. The mince can be stored at –20 °C for several months without any apparent decrease in yields. A 1.5-kg sample of the frozen mince was allowed to thaw overnight at 4 °C. The mince was homogenized in a Waring blender with 2500 mL of 4 mM sodium tricarballylate (Sigma Chemicals) at pH 5.0 containing 0.05 mM disodium dihydrogen ethylenediaminetetraacetate (Na_2H_2edta) and 10 mM 2-mercaptoethanol (both BDH AnalaR). The mixture was centrifuged at 18000*g* for 10 min. The solid residue was discarded, while the supernatant solution was collected and its pH adjusted to 6.2 by addition of solid tris(hydroxymethyl)aminomethane (Tris buffer; Sigma).

The supernatant solution was then placed in a large flask and mechanically stirred. The flask was placed in a bath containing 40% ethanol–water (v/v). Cold ethanol was added gradually to the flask with continuous stirring until a 30% (v/v) concentration was obtained. During the addition of ethanol the temperature was gradually lowered to –6 °C by adding solid CO_2 to the bath. The solid residue was removed by centrifuging the mixture at 18000*g* for 20 min. More ethanol (27%) was added to the supernatant solution, and the temperature was lowered to –10 °C by adding more solid CO_2 to the bath. The mixture was again centrifuged for 20 min. The supernatant solution was discarded while the precipitate was dissolved in ~150 mL of 2 mM tricarballylate–Tris buffer at pH 6.2 (Sigma Chemicals). The resulting protein solution was dialyzed overnight against 4 L of the same buffer.

The dialyzed solution was centrifuged at 40000*g* for 10 min to remove any solid residue. Ammonium sulfate (0.35 g/mL) was added slowly to the clear solution with continuous stirring. The mixture was centrifuged at 40000*g* for 20 min. The solid residue was rejected. More ammonium sulfate (0.25 g/mL) was added to the supernatant solutioun with continuous stirring. Centrifugation of the mixture at 40000*g* yielded a solid precipitate, which was dissolved in 5 mM phosphate buffer (pH 7.4) containing 1 mM DL-dithiothreitol (Sigma), and the solution was dialyzed extensively in the same buffer. After dialysis, the protein was loaded onto a Whatman CM52 cellulose column (Biosystem Ltd.; 5 × 15 cm), equilibrated with phosphate buffer. The column was washed thoroughly with 5 mM phosphate buffer, and the protein was eluted with 25 mM phosphate (pH 7.4). Normally two bands, brown and red, are obtained, of which the first brown band is aconitase.

The aconitase fraction was collected and diluted with 5 mM phosphate buffer until the electrical conductivity was the same as that of 8 mM phosphate, and the mixture was loaded onto a CM Sephadex C-50-120 column (Sigma; 2.4 × 50 cm) equilibrated with 8 mM phosphate buffer. Elution using a linear gradient of 800 mL each of 8 and 25 mM phosphate (pH 7.4) gave fractions containing pure aconitase. The enzyme solution was concentrated, and the buffer was changed to 85 mM *N*-[2-hydroxyethyl)piperazine-*N'*-ethanesulfonic acid (Hepes, pK_a 7.5; Sigma), by Amicon ultrafiltration using a PM-30 membrane. Concentrations of the enzyme were determined spectrophotometrically from the absorbance at 280 nm, $\epsilon = 92\,000\ M^{-1}\ cm^{-1}$.[34,35] Yields of ~120 mg of aconitase were obtained.

Other Reagents. The following were used: ferrous ammonium sulfate, $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$, and sodium sulfate, Na_2SO_4 (both BDH, AnalaR); sodium dithionite, $Na_2S_2O_4$, and manganese perchlorate, $Mn(ClO_4)_2{\cdot}6H_2O$ (both Fluka); iron(II) perchlorate, $Fe(ClO_4)_2{\cdot}6H_2O$; cobalt(II) perchlorate, $Co(ClO_4)_2{\cdot}6H_2O$, and zinc(II) perchlorate, $Zn(ClO_4)_2{\cdot}6H_2O$ (from G. F. Smith Chemical Co.); lithium, copper(II), and nickel(II) perchlorate salts as $LiClO_4{\cdot}3H_2O$, $Cu(ClO_4)_2{\cdot}6H_2O$, and $Ni(ClO_4)_2{\cdot}6H_2O$, respectively (from Aldrich). All chemicals used were as supplied, except the Li^+, Mn(II), and Co(II) perchlorates, which were recrystallized from water. The iron(II) perchlorate was freed from iron(III) impurity by making up the solution at pH 7.4 (85 mM Hepes), when $Fe(OH)_3$ precipitated out and was removed by Millipore filtration. The concentration of Fe(II) was determined by titration against Ce(IV). The concentration of $LiClO_4{\cdot}3H_2O$ solutions was determined by exchanging onto an Amberlite IR(H) 120 cation-exchange column and titrating the H^+ released with standard NaOH. At pH 7.4 and in perchlorate solutions at I = 0.100 M, the M(II) metal ions were assumed to be present as $[M(H_2O)_6]^{2+}$.

Kinetics. All reactions were carried out at 25 °C under air-free conditions. Solutions were prepared in a Miller-Howe glovebox (O_2 < 5 ppm) in air-free 85 mM Hepes buffer at pH 7.4. High concentrations of buffer are essential for proteins such as aconitase having high molecular weights. The ionic strength was adjusted to 0.100 M with lithium perchlorate or sodium sulfate, whichever was appropriate. Reduction of the aconitase $Fe_3S_4^+$ cluster to $Fe_3S_4^0$ was achieved by adding a stoichiometric amount of $S_2O_4^{2-}$. At least a 10-fold excess of edta over aconitase was added to protein solutions before reduction in order to complex any free Fe(II). UV–vis spectra were recorded on either a Shimadzu UV2101PC or a Perkin-Elmer Lambda 9 spectrophotometer. Kinetic runs were monitored at 25.0 ± 0.1 °C on a Dionex D-110 stopped-flow spectrophotometer, interfaced to an IBM PC/AT-X computer using software from On-Line Instrument Systems (Bogart, GA). The reactions were followed by monitoring formation of the $Fe_3MS_4^{2+}$ cluster at 440 nm for M = Fe and at 460 nm for M = Mn and Co. In perchlorate solutions, the conditions $[M^{2+}] > 10$[aconitase] were maintained. At pH 7.4, sulfate is present as SO_4^{2-}, and conditions of $[SO_4^{2-}] > 10$[Fe(II)] and of [Fe(II)] > 10[aconitase] were adopted. The concentration of aconitase used was in the range $(2.0–3.0) \times 10^{-5}$ M. In the case of the perchlorate solutions, ionic strength variations in the range 0.08–0.10 M had little or no effect on rate constants, which remained within the experimental error.

Preliminary Studies. UV–vis spectra of the $Fe_4S_4^0$ and $Fe_4S_4^{2+}$ forms of aconitase alongside inactive $Fe_3S_4^+$ (Figure 1) are in agreement with spectra reported previously.[4] The changes in spectra with Mn^{2+} and Co^{2+} are similar to those for Fe^{2+}, consistent with the formation of

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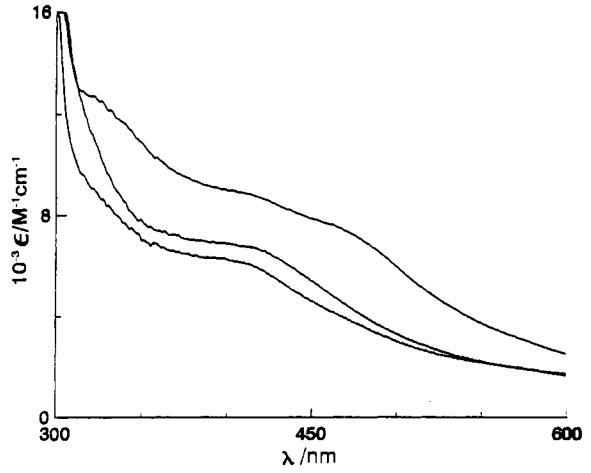

Figure 1. UV–vis absorption spectra of aconitase $Fe_3S_4^0$, $Fe_4S_4^{2+}$, and $Fe_3S_4^+$ (in order lower to upper) at pH 7.4 (85 mM Hepes), I = 0.100 M ($LiClO_4$).

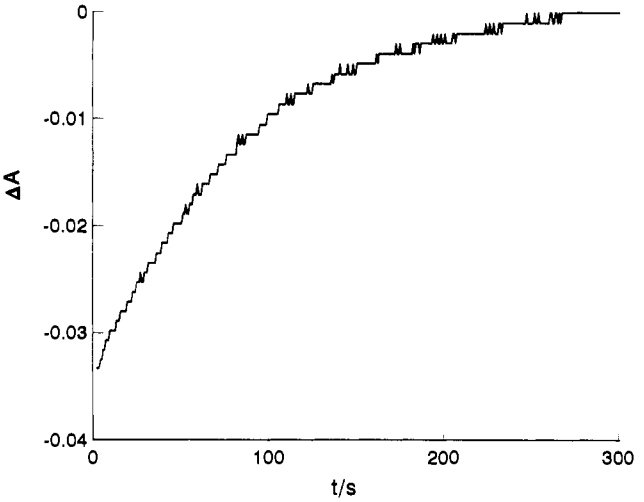

Figure 2. Example of a stopped-flow trace (25 °C), in this case for the reaction of Mn^{2+} (5.0 mM) with aconitase $Fe_3S_4^0$ (0.028 mM) at pH 7.4 (85 mM Hepes), I = 0.100 M ($LiClO_4$).

Table 1. First-Order Rate Constants k_{eq} (25 °C) for the Reaction of Fe^{2+}, Mn^{2+}, and Co^{2+} Hexaaquametal Ions with Reduced Inactive Aconitase $Fe_3S_4^0$ at pH 7.4, I = 0.100 M ($LiClO_4$)

$10^3[Fe^{2+}]/M$	1.50	2.20	3.00	4.0	5.0	6.3	7.5			
$10^3k_{eq}/s^{-1}$	6.2	7.0	8.5	11.6	12.8	15.3	17.4			
$10^3[Mn^{2+}]/M$	1.67	3.00	4.2	5.0	5.0	6.0	7.0	7.5	8.3	9.0
$10^3k_{eq}/s^{-1}$	6.1	7.5	8.6	9.4	10.7	9.8	10.2	12.0	13.4	13.7
$10^3[Co^{2+}]/M$	0.52	0.75	1.00	1.50	2.00	3.00	4.5	5.0	7.0	
$10^3k_{eq}/s^{-1}$	7.9	8.3	10.7	12.8	14.9	23	31	34	45	

$Fe_3MnS_4^{2+}$ and $Fe_3CoS_4^{2+}$. A typical stopped-flow trace is shown in Figure 2. The products can be stable for >24 h under air-free conditions at 4 °C, but this is variable and depends on the concentrations of M^{2+}. Gradual bleaching in the visible range occurs on exposure to air with formation of a white flocculence. Although absorbance changes (ΔA) are small (Figure 1), the magnitude with increasing $[M^{2+}]$ indicates an equilibration process. In the case of Cu^{2+} and Zn^{2+}, precipitation was observed with Hepes buffer.

Results

A single kinetic equilibration step (k_{eq}) is observed for the incorporation of M^{2+} ions (M = Fe, Mn, Co) into aconitase $Fe_3S_4^0$ to give $Fe_3MS_4^{2+}$ products (Table 1). At the higher M^{2+} concentrations, protein denaturation is observed, and this is most marked in the case of Co^{2+}. Thus, at $[Co^{2+}] < 1.5 \times 10^{-3}$ M, the reaction shows satisfactory uniphasic behavior, but at higher concentrations, there is a second phase and, with $[Co^{2+}] > 8 \times 10^{-3}$ M, a white flocculence is observed. Since for midrange $[Co^{2+}]$ runs the denaturation process gives a linear increase in absorbance with time, it was possible by extrapolating back to correct for contributions to the first phase. With this correction, kinetic plots were linear to 2–3 half-lives, and first-order rate constants k_{eq} were obtained (Table 1).

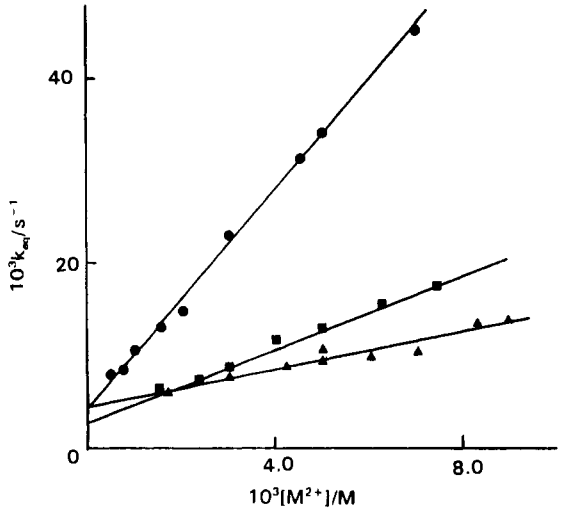

Figure 3. Dependence of rate constants k_{eq} (25 °C) on $[M^{2+}]$ for the equilibration of Fe^{2+} (■) Mn^{2+} (▲), and Co^{2+} (●) with aconitase $Fe_3S_4^0$ at pH 7.4 (85 mM Hepes), I = 0.100 M ($LiClO_4$).

Table 2. Summary of k_1 and k_2 Values (25 °C) for M^{2+} Addition to Aconitase $Fe_3S_4^0$ at pH 7.4, I = 0.100 M ($LiClO_4$) (Errors in Parentheses)

M^{2+}	$k_1/M^{-1}\,s^{-1}$	$10^3k_2/s^{-1}$
Fe^{2+}	1.9(1)	3.2(4)
Mn^{2+}	1.0(1)	4.5(6)
Co^{2+}	5.9(1)	4.4(4)

Table 3. First-Order Rate Constants k_{eq} (25 °C) for the Reaction of Fe(II) with Aconitase $Fe_3S_4^0$ at pH 7.4, in the Presence of Sulfate, I = 0.100 M ($LiClO_4$)

$10^3[Fe(II)]/M$	$10^3[SO_4^{2-}]/M$	$10^3k_{eq}/s^{-1}$
0.6	6.0	5.8
0.9	9.0	8.7
1.0	12.9	12.6
1.0	16.4	14.1
1.0	20.4	15.9

When k_{eq} values (Table 1) are plotted against $[M^{2+}]$, straight lines with a positive intercept are obtained (Figure 3). The rate law can therefore be expressed as in (4). Values of k_1 (slope) and

$$k_{eq} = k_1[M^{2+}] + k_2 \qquad (4)$$

k_2 (intercept) obtained by an unweighted least-squares treatment are listed in Table 2. Equilibrium constants $10^{-2}\,K/M^{-1}$ ($=k_1/k_2$) are 6.1 ± 1.1 (Fe^{2+}), 2.2 ± 0.4 (Mn^{2+}), and 13.5 ± 1.5 (Co^{2+}). Values of similar magnitude were obtained from a consideration of changes in absorbance (A) with $[M^{2+}]$. Thus the expression (5) can be derived, where $\Delta\epsilon$ is the difference in absorption coefficients for the product and reactant (Figure 1). From graphs of the left-hand-side of (5) against $[M^{2+}]^{-1}$ the slope and intercept

$$\frac{[\text{aconitase}]}{A_\infty - A_t} = \frac{1}{K\Delta\epsilon}\frac{1}{[M^{2+}]} + \frac{1}{\Delta\epsilon} \qquad (5)$$

yield $10^{-2}K/M^{-1}$ values of 3.4 ± 1.0 (Fe^{2+}), 1.7 ± 0.3 (Mn^{2+}), and 15.4 ± 3.2 (Co^{2+}). On reacting Ni^{2+} (4.8 mM) with aconitase (43 μM), we observed denaturation with formation of a white flocculence at an early stage, and it was not possible to obtain meaningful k_{eq} or K values.

When the Fe(II) reaction was carried out in the presence of sulfate, higher k_{eq} values were obtained (Table 3). The full rate law dependence is as in (6), with no evidence for significant contributions from the further term $k_4[SO_4^{2-}]$, where k_4 is for the

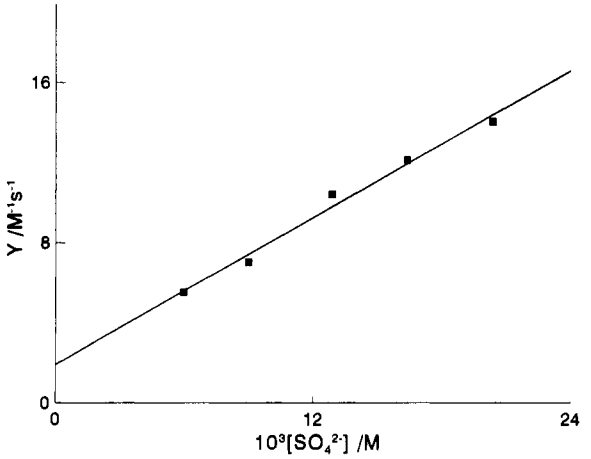

Figure 4. Dependence of rate constants k_{eq} (25 °C) for the equilibration of Fe^{2+} with aconitase $Fe_3S_4^0$ in the presence of SO_4^{2-}: plot of the left-hand side of eq 8, here designated as Y, against $[SO_4^{2-}]$ at pH 7.4 (85 mM Hepes), I = 0.100 M ($LiClO_4$).

$$k_{eq} = k_1[Fe^{2+}] + k_2 + k_3[FeSO_4] \quad (6)$$

reverse of the k_3 reaction. The formation constant for $[Fe(H_2O)_5SO_4]$ can be defined as in (7). For the conditions $[SO_4^{2-}] >$

$$Fe^{2+} + SO_4^{2-} \overset{K_s}{\rightleftarrows} FeSO_4 \quad (7)$$

10[Fe(II)] and [Fe(II)] > 10[aconitase], mass balance [Fe(II)] = $[Fe^{2+}]$ + $[FeSO_4]$ applies, and the expression (8) can be derived.

$$\frac{(k_{eq} - k_2)(1 + K_s[SO_4^{2-}])}{[Fe(II)]_T} = k_1 + k_3K_s[SO_4^{2-}] \quad (8)$$

Assuming k_2 is unchanged in the presence of SO_4^{2-}, a plot of the left-hand side of (8) versus $[SO_4^{2-}]$ gives a straight line (Figure 4), with the intercept corresponding to k_1 = 2.3 ± 0.3 M^{-1} s^{-1}. Using K_s = 2.17 M^{-1}, the slope (k_3K_s) gives k_3 = 276 ± 10 M^{-1} s^{-1}. The value for K_s was obtained from previously reported values of K_{12} = 13 M^{-1} and K_{23} = 0.167 as defined in (9),[36,37] using the relationship $K_s = K_{12}K_{23}$.

$$[Fe(H_2O)_6]^{2+} + SO_4^{2-} \overset{K_{12}}{\rightleftarrows} [Fe(H_2O)_6]^{2+}, SO_4^{2-} \overset{K_{23}}{\rightleftarrows} [Fe(H_2O)_5SO_4] \quad (9)$$

Discussion

Incorporation of the divalent metal ions M^{2+} = Fe^{2+}, Mn^{2+}, and Co^{2+} into the $Fe_3S_4^0$ cluster of aconitase was studied in Hepes buffers (85 mM, pH 7.4). Hepes was chosen because it is reported to provide a noncomplexing medium for divalent metals,[38] rendering the study as simple as possible. All three hexaaqua ions Fe^{2+}, Mn^{2+}, and Co^{2+} are incorporated into the $Fe_3S_4^0$ cluster in an equilibration process, (10), with second-order rate constants

$$Fe_3S_4^0 + M^{2+} \underset{k_2}{\overset{k_1}{\rightleftarrows}} Fe_3MS_4^{2+} \quad (10)$$

k_1/M^{-1} s^{-1} for the formation step of 1.9 (Fe), 1.0 (Mn), and 5.9 (Co) (Figure 3). The narrow span of values is similar to that for the characteristic rate constants of 4.4 × 10^6, 2.1 × 10^7, and 3.2 × 10^6 s^{-1} for H_2O exchange on hexaaqua $Fe^{2+}(t_{2g}^4e_g^2)$, Mn^{2+}-$(t_{2g}^3e_g^2)$, and $Co^{2+}(t_{2g}^5e_g^2)$, respectively,[39] but does not parallel exactly the latter. It is possible to convert k_1 values into first-order rate constants by dividing by an equilibrium constant for association of M^{2+} to one (or more) intermediate sites on the aconitase, in which case small values of order of magnitude 10^{-5} M^{-1} are required. More specifically, the need to access the $Fe_3S_4^0$ cluster in the protein, the multistep nature of the reaction with formation of three M–S bonds, and the likely requirement of a change in the coordination of M^{2+} from octahedral to tetrahedral represent barriers to reaction.

The dissociation rate constants $10^3k_2/s^{-1}$ of 3.2 (Fe), 4.2 (Mn), and 4.4 (Co) obtained from the intercepts enable equilibrium constants $10^{-2}K/M^{-1}$ ($=k_1/k_2$) of 6.1 (Fe^{2+}), 2.6 (Mn^{2+}), and 13.5 (Co^{2+}) to be calculated. Butt et al.[30] have indicated from their incorporation studies on the [3Fe–4S] ferredoxin from *D. africanus* a thermodynamic affinity in the order $Cd^{2+} > Zn^{2+} > Fe^{2+}$ (10^{-4} K values of 125, 63, and 3.3 M^{-1}, respectively). The value obtained for Fe^{2+} is 54 times more favorable in the case of *D. africanus* than for aconitase. This may reflect the easier access of M^{2+} to the cluster which is near the surface of the protein. However, whereas the ferredoxins are very acidic, aconitase is basic with a pI of ~8.5, and charge alone might account for the difference observed. The combined results give an order of equilibrium constants K of $Zn^{2+} > Co^{2+} > Fe^{2+} > Mn^{2+}$, which is the same as that observed in the Irving–Williams order.[40] The latter reflects the increasing inherent acidity of the metal (largely due to the decrease in radius with increasing atomic number), superimposed on which is the hardness–softness factor. The order $Cd^{2+} > Zn^{2+}$ is consistent with the greater softness of Cd^{2+} and its affinity for S^{2-}. It can also be predicted that the Co^{2+} adduct will have less thermodynamic affinity than in the case of Ni^{2+}, which may relate to the difficulty encountered with Ni^{2+} and its tendency to denature aconitase. Holm and colleagues[21] in their analogue studies have also commented on the relative instability of Co- and Ni-containing Fe_3MS_4 clusters. The activity of aconitase in the presence of metal ions other than Fe^{2+} has been tested but none has been observed.[41]

In the aconitase X-ray crystal structure determination, a single sulfate was found to be incorporated in the active site pocket.[42] This prompted us to study the Fe^{2+} incorporation in the presence of sulfate. Rate constants k_{eq} increased markedly. The effect was quantified by including a $[SO_4^{2-}]$-dependent term in the rate law (8) corresponding to $[Fe(H_2O)_5SO_4]$ participation. The second-order rate constant k_3 for the $[Fe(H_2O)_5SO_4]$ reaction of 276 M^{-1} s^{-1} is 140-fold that for Fe^{2+}, which suggests that the $Fe_3S_4^0$-containing pocket of aconitase is more accessible to neutral as opposed to positively charged reactants. Apart from the more favorable overall electrostatics with $[Fe(H_2O)_5SO_4]$, histidine residues His-101, His-147, and His-167 are close to the site normally occupied by Fe_a and could be influential if there is protonation.[1,43] Such positively charged residues will act as a further deterrent to Fe^{2+} but not to $FeSO_4$. An alternative explanation is that the Fe^{2+} associates with SO_4^{2-} already present in the aconitase pocket. Although SO_4^{2-} has been detected in the X-ray structure, the crystals were obtained from saturated $(NH_4)_2SO_4$, and it has not been demonstrated that, at the much lower $[SO_4^{2-}]$ as in these studies, sulfate is present at the site so defined. Moreover, SO_4^{2-} may not be very strategically placed for reaction with the $Fe_3S_4^0$ core. Therefore, apart from the fact that sulfate and presumably other anions can assist Fe^{2+} to access the active site of aconitase, no other function or significance seems likely.

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