Relationship of the Oxidation State of the Iron–Sulfur Cluster of Aconitase to Activity and Substrate Binding†

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ABSTRACT: It is known that aconitase from mammalian mitochondria is only partially active as isolated but may be activated by incubation with iron, ascorbate, and a thiol, or with dithionite. It has been suggested that the added Fe in the activation mixture is essential for activation and that it is incorporated in the enzyme [Villafranca, J. J., & Mildvan, A. S. (1971) *J. Biol. Chem. 246*, 772–779; Gawron, O., Waheed, A., Glaid, A. J., & Jaklitsch, A. (1974) *Biochem. J. 139*, 709–714]. However, it is shown in this paper that, when the enzyme has a full complement of 3Fe and 3S, full activation is reached coulometrically, without iron or other chemical reducing agents. It is clear, therefore, that the role of activators is to reduce the iron–sulfur cluster of the enzyme. The appearance of catalytic activity on reduction of the cluster shows a pronounced lag, as does the decay of activity after reoxidizing the cluster. This suggests that catalytic activity requires a conformational change in the protein which is initiated by reduction of the cluster and that, following reoxidation, activity disappears only after the inactive conformation is assumed. Citrate and the competitive inhibitor *trans*-aconitate are bound to a comparable extent to the active and inactive forms, but only the active form can bind 1-hydroxy-2-nitro-1,3-propanedicarboxylic acid, a transition-state analogue. This is interpreted to show that in the inactive state aconitase cannot enter the conformation it assumes in the transition state during catalysis.

It is now well-known that although aconitase (EC 4.2.1.3) does not catalyze a redox reaction, it contains an iron–sulfur cluster (Ruzicka & Beinert, 1978a). This recognition has cast doubt on catalytic mechanisms involving Fe^{2+}, such as the ferrous wheel mechanism [for a review, see Villafranca (1974a)], and raised questions about the possible mechanistic or regulatory role of the cluster in aconitase. The iron–sulfur center, now thought to be a [3Fe–3S] cluster (Emptage et al., 1980; Kent et al., 1981), is reduced and electron paramagnetic resonance (EPR) silent in the active enzyme (Ruzicka & Beinert, 1978a). When the enzyme is oxidized, an EPR signal, centered at $g = 2.01$, until then considered typical of [4Fe–4S] clusters in the 3+ oxidation state (i.e., corresponding to oxidized high-potential Fe–S proteins), appears, and the catalytic activity is lost.

Aconitase is usually only partially active as isolated but may be activated by incubation for 10 min at room temperature with iron, ascorbate, and a thiol (Gawron et al., 1974) or by irradiation in the presence of deazaflavin (Ruzicka & Beinert, 1978b). Activation also occurs after reduction by dithionite, although to a somewhat lesser extent, but reoxidation thereafter yields inactive enzyme with a slightly different visible spectrum and a different EPR signal shape and intensity (Beinert et al., 1979). The change in activity following reduction by dithionite (or reoxidation thereafter) is not immediate but occurs slowly after the redox change (Beinert et al., 1979). We shall report in this paper that the lag is also observed during redox cycling of aconitase by coulometric titration. This behavior suggests that cluster reduction (or oxidation) is a requirement for activation (or deactivation) of the enzyme and that the change in oxidation state is then followed by a slow conformational change to the active (or inactive) form of the enzyme.

Experiments which concern details of the activation process, such as its dependence on the reagents or combination of reagents used, on the presence of oxygen, trace iron impurities, or chelating agents, and on time and temperature, will be the subject of a future report.[1]

To assess possible structural differences between the oxidized and reduced forms of aconitase, we have studied the effect of the oxidation state of the iron–sulfur cluster on the binding of substrates and inhibitors to the enzyme. The nitro analogue of isocitrate (a competitive inhibitor) is of particular interest, because the initial enzyme–inhibitor complex undergoes a slow change leading to tighter binding (Schloss et al., 1980). The very low dissociation constant for 1-hydroxy-2-nitro-1,3-propanedicarboxylic acid (nitroisocitrate)[2] is consistent with it being a transition-state analogue, and its structure in the ionized form closely resembles the carbanion derived from isocitrate. Here we present evidence that a slow conformational change (distinct from that postulated to occur following reduction of the iron–sulfur cluster to generate the active form of aconitase) is responsible for the increase in affinity for nitroisocitrate and that oxidized aconitase is incapable of this conformational change, although it binds the substrate with about the same affinity as the reduced species.

Materials and Methods

Enzyme Preparations. Aconitase was prepared from beef heart mitochondria as described by Ruzicka & Beinert (1974)

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[1] M. C. Kennedy, J.-L. Dreyer, and H. Beinert, unpublished experiments.

[2] Abbreviations used: DTT, dithiothreitol; DTE, dithioerythritol; NEM, *N*-ethylmaleimide; $NaDodSO_4$, sodium dodecyl sulfate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); nitroisocitrate, 1-hydroxy-2-nitro-1,3-propanedicarboxylic acid; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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except that the preparations used for coulometric titrations and binding studies were kept anaerobic at all times during isolation. The anaerobic preparations contained 2.5–2.9 mol of iron per mol and the same amount of labile sulfur. Specific activities were between 7.4 and 8.3 μmol min^{-1} mg^{-1} after activation. The preparations were at least 95% pure, as judged by $NaDodSO_4$ gel electrophoresis, and the molecular weight was 83000, as found by Kurtz et al. (1979). Aconitase activity was assayed by using a coupled assay (Rose & O'Connell, 1967) or by the appearance of aconitate (Schloss et al., 1980). The maximum activity was measured after activation according to Gawron et al. (1974). Non-heme iron was determined according to Brumby & Massey (1967) and labile sulfur by the method of Lovenberg et al. (1963). For EPR studies and those involving reoxidation by cobalt(III) phenanthroline, preparations were isolated, assayed, and activated as described (Beinert et al., 1979).

Cytoplasmic aconitase was prepared from beef liver by the method of Henson & Cleland (1967) and further purified by chromatography on DEAE-cellulose (Guarriero-Bobyleva et al., 1973) and by filtration through Sephacryl S-200. The activity was 0.81 unit mg^{-1} after activation with iron and cysteine (Rose & O'Connell, 1967). The enzyme retained a specific activity of 0.22 unit mg^{-1}, so was used without activation in the kinetic studies.

Synthesis of Radiolabeled Inhibitors. The nitro analogue of isocitrate was synthesized from [^{14}C]oxalic acid purchased from New England Nuclear. [^{14}C]Glyoxylic acid was prepared from [^{14}C]oxalic acid (44 μmol, 250 μCi) by the electrolytic method of Krupka & Towers (1958). [^{14}C]Glyoxylate (42 μmol) was condensed with nitropropionate (56 μmol) in the presence of excess sodium carbonate (100 mg, 1 mL of water) for 30 min and the resultant 1-hydroxy-2-nitro-1,3-[^{14}C]propanedicarboxylate (23 μmol) purified by silica gel chromatography (Schloss et al., 1980). Before use, the radiochemical purity was assessed on high-performance thin-layer chromatography plates of silica gel (SG 60F-254) with fluorescent indicator from E. Merck, Darmstadt, Germany, developed in 1-butanol–acetic acid–water (10:5:8). The spots, visible under shortwave ultraviolet light, were scraped off and mixed with water, and the radioactivity was determined. The R_f value for the nitro inhibitor was 0.55, while that for the contaminant was 0.70. The contaminant was 12% of the total radioactivity and was identified as glyoxylate by assay with lactate dehydrogenase.

[^{14}C]Citrate (specific activity 96 Ci mol^{-1}) was obtained from New England Nuclear. A mixture of anhydrous [1,5-$^{14}C_2$]citric acid (0.31 μmol, 75 μCi), 30 μL of water, and 33 μL of concentrated sulfuric acid was incubated at 143 °C for 9 h in a sealed tube in vacuo (50 μmHg, N_2 atmosphere) to convert the citrate to *trans*-aconitate (Bruce, 1943). The resultant [^{14}C]aconitate (0.11 μmol) was purified by silica gel chromatography as described by Bulen et al. (1952) and by elution from a water-washed DEAE-cellulose column (18 × 2 cm) with a 400-mL linear gradient of from 20 to 50 mM KCl. The pure product (specific activity 0.26 Ci mol^{-1}) was identical with *trans*-aconitate in thin-layer chromatography.

Coulometric Redox Cycling. Aconitase (to give 3.3 mg of protein per mL) was added anaerobically to 50 mM $NaPO_4$ (pH 7.0) buffer containing 1 mM methyl viologen, 1 mM $K_4Fe(CN)_6$, 10 μM indigotetrasulfonate, 10 μM anthraquinone-1-sulfonate, and 10 μM 2,3-dimethoxy-5-methyl-6-*n*-pentyl-1,4-benzoquinone (DPB), and the mixture was loaded anaerobically into the coulometric cell (path length 1 cm). The cell and its operation were as described by Wilson (1978). Active (reduced) or inactive (oxidized) aconitase could then be transferred to anaerobic vials for other experiments.

Activated aconitase for the binding experiments was also prepared by anaerobic incubation at 17 mg mL^{-1} in 33 mM Hepes (pH 7.0) containing 20 mM ascorbate, 2.8 mM $FeSO_4$, and 2.8 mM DTE (Gawron et al., 1974). After 10 min at room temperature, the mixture was diluted to 2 mg mL^{-1} with anaerobic 50 mM Tris-HCl (pH 8.0) at 4 °C. This stock solution was assayed for activity and protein, and aliquots were taken for experiments as described in the figure legends. Oxidized aconitase was prepared by removing DTE on a G-25 (fine) column and exposing the enzyme to oxygen overnight or for 6 h at 4 °C. Residual activity and the reactivated activity were measured. Alternatively, an 8-fold excess of $Fe(CN)_6^{3-}$ was added to the enzyme anaerobically at room temperature. After 2 h, activity without prior activation was virtually zero, and the enzyme usually could not be fully reactivated.

Binding of the Nitro Analogue of Isocitrate to Aconitase. Enzyme was incubated anaerobically with ^{14}C-labeled nitro inhibitor overnight at room temperature to allow equilibration. Duplicate aliquots (100 μL) of each sample were applied to anaerobic columns of Sephadex G-50 (fine) (packed volume 0.8 mL at 4 °C) prepared according to Penefsky (1977) and centrifuged anaerobically for 2.5 min. For low concentrations of inhibitor, effluents from three columns were pooled for each sample. The columns were then washed first with 100-μL and then 3 times with 400-μL aliquots of buffer. The radioactivity was determined in all fractions.

Equilibrium dialysis of aconitase (4 mg mL^{-1}) with citrate was conducted anaerobically in 50 mM Tris-HCl (pH 8.0) containing 1 mM DTE and 2 mM EDTA at room temperature for 16 h, using a microdialysis unit (Hoeffer Scientific Instruments, 0.27 mL per compartment). Donnan effects were minimized by adding 0.15 M KCl. Binding constants were derived by nonlinear regression using a computer program (Duggleby, 1981).

Measurement of Thiols. Aliquots of aconitase (to give 0.64 mg mL^{-1}, 7.7 μM) were added to anaerobic cuvettes containing 50 mM Tris-HCl (pH 8.0) and 0.33 mM DTNB at 30 °C, and the reaction was followed at 412 nm in a Cary 219 spectrophotometer. The entire experiment was conducted under anaerobic conditions. Alternatively, aliquots (1 mL, 0.69 mg of protein) were anaerobically reacted with 0.1 mM [^{14}C]NEM (2.45 Ci mol^{-1}). At given times, aliquots (180 μL) were removed and added to 200 μL of 20 mM DTT to stop the reaction. The protein was precipitated in 10% (w/v) trichloroacetic acid, washed with 1 mL of 5% (w/v) trichloroacetic acid, and then redissolved in 1 mL of 10 mM sodium phosphate, pH 7.0, containing 1% (w/v) $NaDodSO_4$ and mercaptoethanol. Beckman GP scintillation fluid (10 mL) was added and the radioactivity determined.

Results

Activation and Deactivation of Aconitase. Beinert et al. (1979) have previously reported on the complex behavior of aconitase during activation by dithionite, followed by reoxidation with O_2. It was noted that on reduction with a limited quantity of dithionite, disappearance of the EPR signal of the oxidized enzyme is rapid, as is partial (~50%) bleaching of the absorbance of the Fe–S cluster at 480 nm, but the appearance of enzyme activity is slow. On admission of O_2, the absorbance at 480 nm and the EPR signal were only partly restored, but activity remained at nearly the level found after dithionite treatment and decayed only very slowly. All this suggests that activation and its reversal may be the results of

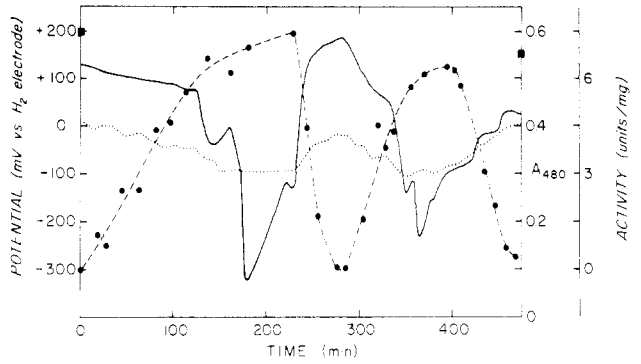


FIGURE 1: Time course of coulometric redox cycling of aconitase. The procedure is described under Materials and Methods. The potential was varied as shown by the solid line. The change in absorbance at 480 nm (dotted line) was monitored and the activity (dashed line) measured at intervals. The squares indicate the maximum activity after activation with iron, thiol, and ascorbate.

a structural change in the protein initiated by altering the redox state of its Fe–S cluster.

These findings were complicated by apparently irreversible changes associated with reduction by dithionite. Thus, at protein concentrations in the micromolar range, the recovery of the EPR signal on oxidation was close to nil, but at 100 μM or higher concentrations, up to 80% of the EPR signal was recovered. At low protein concentration, the presence of bovine serum albumin during reduction improved the yield of the EPR signal on subsequent exposure to O_2. Oxidation by ferricyanide instead of O_2 led to destruction of part of the Fe–S cluster. Tris(phenanthroline)cobalt(III) chloride was found to be a more suitable oxidant.

In the present study, these observations were followed up by monitoring the oxidation state of the Fe–S cluster by EPR during anaerobic reoxidation of the cluster by cobalt(III) tris(phenanthroline)·$7H_2O$ (Pfeiffer & Werdelmann, 1950). The enzyme was activated by a 2-fold molar excess of dithionite over the enzyme in a closed anaerobic cell with several side arms. Cobalt(III) phenanthroline was then added anaerobically either before or after tipping the activated enzyme into the cuvette part of the cell which contained the assay mixture (Rose & O'Connell, 1967). In either case, the enzyme initially displayed its maximum activity. The activity, however, slowly declined after addition of the oxidant. When the activated enzyme is added to the assay mixture first, the activity achieved by the activation could be briefly monitored before adding the oxidant. On the other hand, in this version of the experiment, it was impossible to record an EPR signal from the reoxidized enzyme because of the dilution in the assay mixture. The alternative version, in which the oxidant was added after activation but before tipping the enzyme into the assay mixture, allowed us to establish that the Fe–S cluster of the enzyme was indeed reoxidized before the enzymatic activity was monitored. In this latter version of the experiment, it was not possible to determine enzymatic activity of the same sample before adding the oxidant. Both sets of experiments together, however, established that while the Fe–S cluster is immediately oxidized by cobalt(III) phenanthroline, enzymatic activity persists for periods in the range of minutes to hours.

In order to avoid addition of any oxidizing or reducing agents, we studied changes in absorbance and activity during a reversible redox titration in a coulometric cell. Coulometric cycling only requires the presence of electrochemical mediators. Figure 1 shows the time course for two redox cycles of aconitase. After the first reduction, the activity was equal to that found after activation with iron, ascorbate, and DTE. The

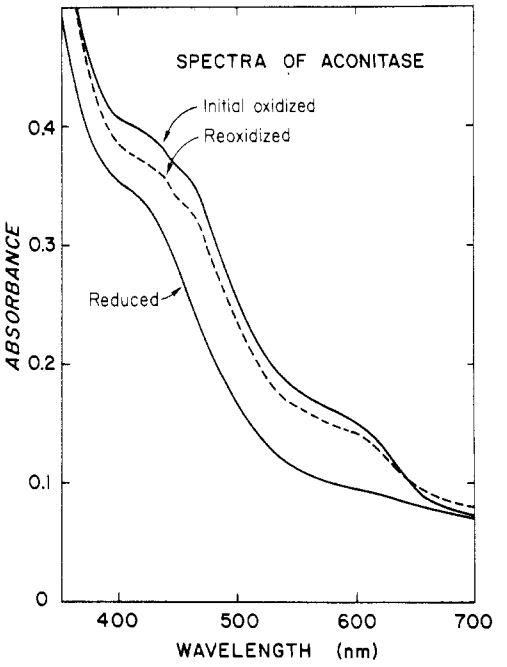


FIGURE 2: Spectra of aconitase during the course of coulometric cycling. The spectra were recorded during the experiment shown in Figure 1 at t = 0 (initial oxidized), 200 (reduced), and 280 min (reoxidized).

experiment clearly shows that neither added iron nor thiols are absolute requirements for converting aconitase into an active form.

When aconitase was reoxidized and then reduced for a second time, the maximum activity attained was lower than that after the first reduction. The reason for the decrease is not apparent, but it has been suggested that some of the relatively fragile iron–sulfur centers could undergo an irreversible change during the redox cycle (Beinert et al., 1979). Similarly, the absorption spectrum of the reoxidized enzyme differs from the initial oxidized spectrum (Figure 2). As noted above, incomplete reversibility was also observed by EPR for aconitase reoxidized by aeration after reduction by dithionite or other reducing agents (Beinert et al., 1979).

The reduction and oxidation of the iron–sulfur center of aconitase were followed at 480 nm. These absorbance changes take place in the potential range −50 to +150 mV. Changes in potential outside that range are due to titration of the dyes. When the absorbance changes during coulometric cycling were plotted against potential, a pronounced hysteresis was observed; i.e., the curve obtained during reoxidation differed significantly from that found during reduction (not shown). This precluded calculation of the midpoint potential of the iron–sulfur cluster from such data.

As shown in Figure 1, in the range of −50 to +150 mV, in which oxidation–reduction of the cluster seems to occur, the appearance of full catalytic activity lagged considerably behind the potential and the absorbance changes associated with the Fe–S cluster. Similarly, during coulometric reoxidation, the decline of activity occurred slowly after the potential was established. This delay is not due to slow equilibration with mediator dyes, because the changes in the absorbance of Fe–S cluster were instantaneous. These observations are in accord with the previous finding (Beinert et al., 1979) that the appearance or decay of the EPR signal was instantaneous during chemical oxidation and reduction, while changes in activity followed more slowly. The simplest explanation of the hysteresis is that a change in the redox state of aconitase leads to a slow conformational change in the protein, which affects

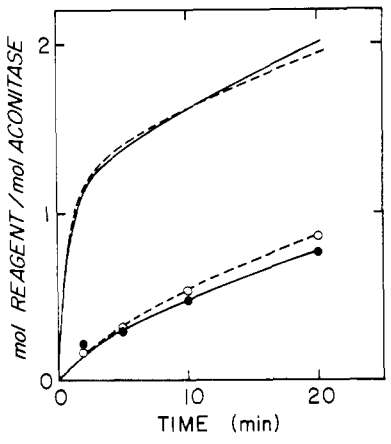


FIGURE 3: Measurement of thiols in active and inactive aconitase. Aconitase was passed through Sephadex G-50 to remove DTE. The upper curves show titration with DTNB of the enzyme (0.64 mg mL^{-1}) activated with 5 mM iron, specific activity 3.28 μmol min^{-1} mg^{-1} (---), or inactivated by exposure to oxygen (—). The lower curves show incorporation of *N*-ethyl[2,3-$^{14}C_2$]maleimide into aconitase (0.69 mg mL^{-1}) coulometrically reduced, specific activity 4.0 μmol min^{-1} mg^{-1} (○), or oxidized (●).

the activity. For the enzyme to assume the active conformation, the iron–sulfur cluster must first be reduced.

Reactive Sulfhydryl Group of Aconitase. Johnson et al. (1977) showed that phenacyl bromide reacted with only one thiol group of inactive aconitase and that this modification prevented subsequent activation of the enzyme. Further, citrate and tricarballylate protected against modification by phenacyl bromide (the former in the active, the latter in the inactive enzyme), so they concluded that an essential thiol group was present at the active site. Preliminary evidence (Beinert et al., 1979) suggested that the number of thiol groups reacting rapidly with thiol reagents increased after reduction of the enzyme. However, these results were not reproducible and were most likely due to traces of DTT in the particular enzyme preparation, which had been carried over from early purification steps. The oxidized form of DTT may have been reduced on chemical activation, leading to an enhanced reaction with DTNB.

Since the appearance of an –SH group on activation would be an important clue to the chemical basis of the activation–deactivation cycle, we decided to examine this question by titrating aconitase with DTNB and by measuring the binding of *N*-[^{14}C]ethylmaleimide. Figure 3 summarizes experiments in which the reactive –SH group titer was determined by both of these methods in the active and inactive forms of the enzyme. No difference was found in the number or reactivity of thiol groups in the two forms. Thus, the conformational change involved in activation does not increase the reactivity of a buried thiol group nor is it dependent on reduction of a disulfide bridge. It may be mentioned in this context that attempts to activate the enzyme via the thioredoxin–thioredoxin reductase system of calf liver (Buchanan et al., 1979) were unsuccessful. We were also unable to inhibit aconitase by 2,3-dimercaptopropanol plus arsenite or arsenite alone, reagents that have been shown to inactivate vicinal dithiols (Carlson et al., 1978). It is also of interest to mention that the EPR signal of the Fe–S cluster is not significantly changed in shape or size after inactivation of the enzyme by *N*-ethylmaleimide.

Binding of Substrates and Analogues to Aconitase. Since no evidence has been found that the activation of aconitase involves the appearance of a functional group required for catalytic activity, the possibility was investigated that a conformation change occurs during activation which increases the affinity of the enzyme for its substrates. The binding of citrate to oxidized and to activated aconitase was measured by equilibrium dialysis to test this point. Since residual active enzyme (0.18 μmol min^{-1} mg^{-1}) remained in the oxidized sample and the activated enzyme is, of course, fully active, the [^{14}C]citrate was converted during the dialysis to the equilibrium mixture of citrate (88.5%), *cis*-aconitate (4.1%), and isocitrate (7.5%) (Glusker, 1971). Considerable scatter in experimental points was inevitable because enzyme concentrations close to the anticipated K_D (100 μM) are impractical. Nevertheless, major differences in the affinity of oxidized and activated aconitase for substrate would be detected.

Activated aconitase (4 mg mL^{-1}, 48 μM), which had retained only 28% of the maximal activity after dialysis, still bound 0.7 mol of substrate per mol of enzyme, with an apparent dissociation constant of 131 μM. Binding to oxidized aconitase (residual specific activity, 0.18 μmol min^{-1} mg^{-1}) was similar: 0.5 mol of substrate was bound per mol of enzyme, with an apparent dissociation constant of 133 μM. Thus, no gross difference in the binding of substrates to the two forms of the enzyme was detected.

An alternative and more accurate method for comparing the affinities of the active and inactive forms for substrates is the use of a competitive inhibitor, such as *trans*-aconitate, which, unlike citrate, is not metabolized in the course of the equilibrium dialysis. Although there is a consensus that inhibition by *trans*-aconitate displays competitive features, literature reports disagree as to whether it is a purely competitive (Gawron & Jones, 1977) or a mixed inhibitor with respect to isocitrate (Villafranca, 1974b). We therefore determined the kinetics of the inhibition of the mitochondrial enzyme from beef heart and found it to be purely competitive with respect to isocitrate, with a K_I value of 208 μM at 30 °C and pH 8, in good agreement with Schloss' data[3] for the cytoplasmic enzyme from beef liver.

Experience in this laboratory has shown that considerable inactivation occurs during the long period of equilibrium dialysis when binding to the reduced, active form is being measured, although the oxidized, inactive form is quite stable under these conditions. We decided, therefore, to compare the K_D value of the oxidized form for *trans*-aconitate with the K_I value of the reduced enzyme. Equilibrium dialysis of aconitase, oxidized by exposure to O_2 (3.67 mg mL^{-1}, specific activity = 0.03 μmol of NADH min^{-1} mg^{-1}), gave a binding constant $K_D = 223 \pm 33$ μM at pH 8 and 25 °C. The sample of oxidized enzyme used could still be activated to 63% of its original activity and bound 0.66 mol of *trans*-aconitate per mol. The satisfactory agreement of this K_D value with the K_i of 208 μM reported above strongly suggests that the binding site for *trans*-aconitate, and, hence, presumably for the substrate, is not significantly changed by oxidation–reduction of the Fe–S cluster.

Binding of Transition-State Analogues to Aconitase. The availability of nitroisocitrate, a transition-state analogue and inhibitor of aconitase (Schloss et al., 1980), in radioactive form permitted the extension of these experiments to the binding of nitroisocitrate by active and inactive aconitase, in an attempt to ascertain whether the structural difference might not be concerned with the transition state of the enzyme.

The nitro analogue of isocitrate is a slow-binding competitive inhibitor of cytoplasmic aconitase. Mitochondrial aconitase exhibited a slow, gradual increase in inhibition by the nitro

[3] K_I = 192 μM at 25 °C; J. V. Schloss, unpublished experiments.

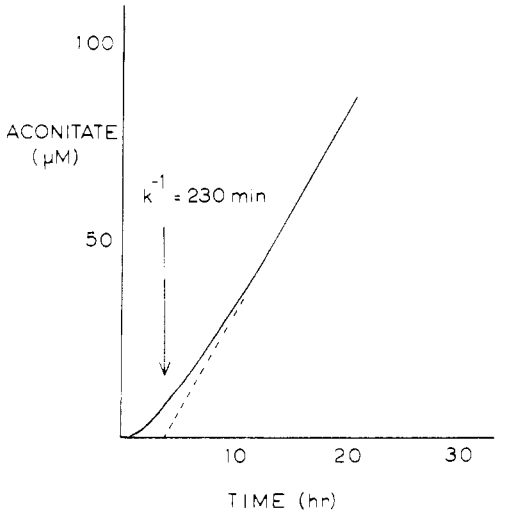


FIGURE 4: Determination of the rate constant for release of the nitro analogue of isocitrate from the mitochondrial aconitase at 6.5 °C. The enzyme (5.4 μM) was preincubated anaerobically with 30 μM inhibitor followed by 12000-fold dilution (final concentrations: enzyme = 450 pM; inhibitor = 2.5 nM) into an assay containing 20 mM DL-isocitrate (K_m = 35 μM).

analogue similar to that seen with the cytoplasmic enzyme (Schloss et al., 1980). On the basis of the final steady-state inhibition of the beef heart mitochondrial enzyme by 0.3 mM inhibitor in the presence of 20 mM DL-isocitrate (in 0.1 M Tris buffer, pH 8.0), a K_I of 12 nM was calculated, the same as that for the cytoplasmic enzyme in liver. When the experiment was repeated by preincubating the enzyme with 1 μM nitroisocitrate and assaying samples at intervals, a range of K_I values (10–47 nM) rather than a precise value was obtained, because in experiments of this type the inhibitor also protects the enzyme against inactivation by O_2. Nevertheless, these results are consistent with the value of 12 nM obtained in assays initiated with aconitase.

To investigate the nature of the slow-binding inhibition seen with both cytoplasmic and mitochondrial aconitases, we determined the rate of release of the inhibitor on dilution at several temperatures. A lag before reaching a steady-state rate was observed for the mitochondrial enzyme, as for the cytoplasmic one (Schloss et al., 1980). Figure 4 shows the lag at 6.5 °C before attaining the linear steady-state rate, equal to that of the uninhibited enzyme. At 25 °C, the rate constant for inhibitor release [calculated from the initial and final slopes of the progress curve, such as shown in Figure 4, as described by Schloss et al. (1980)] was 0.058 min^{-1} for the mitochondrial enzyme and 0.079 min^{-1} for the cytoplasmic one, in reasonable agreement with the rate constant of 0.063 min^{-1} previously reported (Schloss et al., 1980).

An Arrhenius plot for the dissociation data is shown in Figure 5. Activation energies for the release rate of the nitro analogue from cytoplasmic and mitochondrial aconitases are 23.2 and 21.0 kcal mol^{-1}, respectively. The change in the catalytic rate with isocitrate as substrate (data not shown) as a function of temperature also indicated high activation energies. The values obtained were 16.5 kcal mol^{-1} for cytoplasmic aconitase and 19.3 kcal mol^{-1} for the mitochondrial enzyme.

The slow reversal of the tight binding of the nitro inhibitor was exploited in binding experiments, such as that shown in Figure 6. After incubation overnight of enzyme with nitro inhibitor, aliquots were passed down anaerobic Penefsky columns at 3 °C as described under Materials and Methods, in order to separate bound and free radioactivity. Figure 6

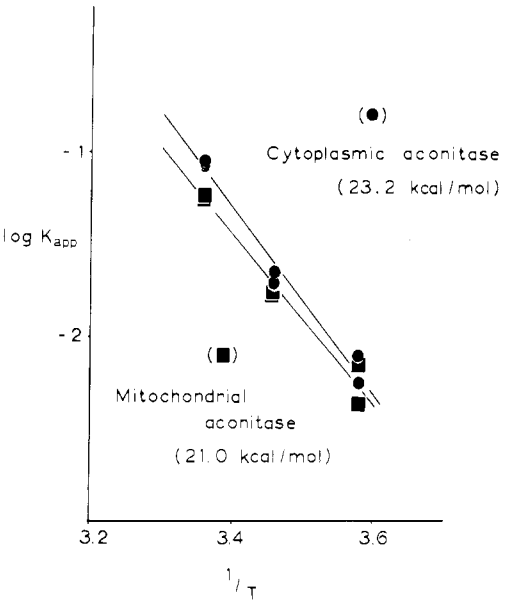


FIGURE 5: Arrhenius plot for the dependence of the rate of release of nitroisocitrate from aconitase on temperature. Dependence of the log of the release rate (K_{app}, min^{-1}) from mitochondrial (■) and cytoplasmic (●) aconitases on the reciprocal (×10^3) of the absolute temperature. Experimental conditions for the cytoplasmic enzyme were as described by Schloss et al. (1980); conditions for mitochondrial aconitase were as described in Figure 4.

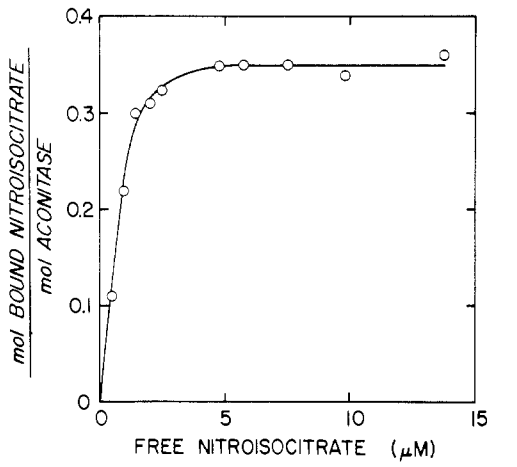


FIGURE 6: Binding of nitroisocitrate to aconitase. The procedure is described under Materials and Methods. Aconitase was activated with iron, thiol, and ascorbate. After dilution to 0.05 mg mL^{-1} (0.6 μM) and anaerobic incubation at room temperature to allow equilibration with the nitro analogue, the residual activity of a control sample was 38% of the full activity. The free inhibitor concentration is based on the free radioactivity without correction for impurities.

is a plot of the amount of nitro inhibitor bound to activated aconitase against the amount of ligand present during the incubation. Only 11.5% of the added radioactivity was bound. The reasons for this are that the stock solution contained 12% impurity and, of the remainder, only one of the four isomers of the nitro inhibitor, namely, 1(*R*)-hydroxy-2(*S*)-nitro-1,3-propanedicarboxylic acid, is likely to be tightly bound (Schloss et al., 1980). However, inhibition is predominantly due to the ionized aci-acid form of the nitro analogue, in which C-3 is trigonal and no longer asymmetric, so that at pH 8 the inhibitor is racemic and the expected binding would be 44%. The discrepancy suggests that the impurities are greater than we had measured, possibly because of the presence of decomposition products which would run with the nitro inhibitor on thin-layer chromatography. Similar breakdown during the overnight incubation is also likely. (Consistent with such

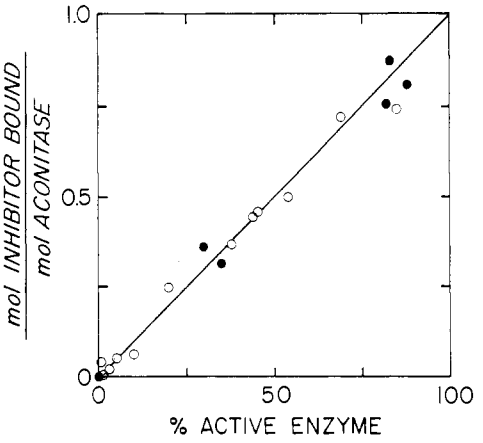


FIGURE 7: Correlation of the amount of the nitro analogue of isocitrate bound to aconitase with the proportion of active enzyme in the incubation. Coulometrically titrated aconitase (1 mg mL^{-1}) was incubated anaerobically overnight with the nitro analogue of isocitrate in a microdialysis unit as described for citrate binding under Materials and Methods (●). Activated or oxidized aconitase (2–50 μg mL^{-1}) was incubated anaerobically with inhibitor overnight and binding determined after centrifugal gel filtration (see Materials and Methods) (○). After the overnight incubations, activities of control samples (no inhibitor) were measured and expressed as a percentage of the maximum activity after activation with iron, ascorbate, and DTE.

breakdown, the nitro analogue, when freshly prepared, contained 5% glyoxylate, which increased to 23% after incubation overnight at room temperature at pH 6.) Saturation of the enzyme was nevertheless achieved, and the shape of the binding curve is consistent with a dissociation constant of about 1% of the enzyme concentration. Thus, the estimated dissociation constant is 5 nM, which agrees well with the K_I (corrected for the noninhibitory isomer). The low specific activity (3.3 Ci mol^{-1}) of the nitro inhibitor and the extremely low K_D preclude the more orthodox calculation of the dissociation constant from a Scatchard plot.

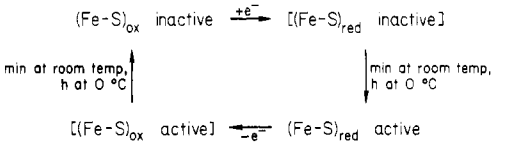
Figure 7 compares the amount of ligand bound to active or inactive enzyme with the residual activity of control samples after the incubation. The active aconitase binds the nitro inhibitor in proportion to its residual activity, so that extrapolation to 100% activity gives a value of 1 mol of inhibitor bound per mol of aconitase. Oxidized aconitase preparations do not bind the inhibitor tightly. If the inhibitor binds to the oxidized form at all, its dissociation constant must be greater than 100 μM, the highest concentration of inhibitor used in equilibrium dialysis experiments.

Thus, there is a clear-cut difference between oxidized and activated (reduced) aconitase: only the latter can bind the nitro analogue and, hence, the transition-state form of the substrate tightly. In other words, it seems that only the active enzyme can undergo the conformational change necessary for the tight binding.

Discussion

Until now, the roles of the various components of the conventional activation mixture (Gawron et al., 1974) have not been clearly defined. The added iron has been assumed to be integrated into the structure (Villafranca & Mildvan, 1971; Gawron et al., 1974). Evidence has recently been obtained to indicate that the labile [3Fe–3S] cluster of aconitase is not only readily converted to a [2Fe–2S] cluster under conditions of extrusion (Kurtz et al., 1979; Beinert et al., 1979, 1981) but also that [3Fe–3S] clusters can occur in different electronic states both in the oxidized and in the reduced forms and that these states are interconvertible (H. Beinert, J.-L. Dreyer, M. C. Kennedy, E. Münck, T. A. Kent, and B. H. Huynh, unpublished experiments). It seems possible that during such interconversions extraneous iron may be exchanged with cluster iron or may enter iron-deficient clusters. However, as shown above, with aconitase which already contained nearly 3 mol of iron and sulfur per mol (Figure 1), added iron was not required for full activity. It is also possible that the ferrous ion of the activation mixture has a catalytic role in the conversion of aconitase into its active conformation.

Scheme I: Relationship of Oxidation State of Fe–S Cluster in Aconitase and Enzyme Activity[a]



[a] The principal forms are $(Fe\text{–}S)_{ox}$ inactive and $(Fe\text{–}S)_{red}$ active, which are stable until a redox change occurs. The forms $(Fe\text{–}S)_{ox}$ active and $(Fe\text{–}S)_{red}$ inactive exist only transiently following oxidation and reduction of the iron–sulfur cluster, respectively.

The experiments presented in this paper suggest that aconitase exists in at least two different forms, transition between which is controlled by the redox state of the iron–sulfur cluster (Scheme I). When the iron–sulfur cluster is reduced, aconitase has the capability to become fully active, but the full activity is observed only after a lag. The simplest explanation is that the rate-limiting step in regaining activity is a relatively slow conformational change which occurs as a result of cluster reduction and is reversed after oxidation.

Substrates (=products) have no effect on the rate of activation, and, as shown in this paper, they bind equally well to the active and inactive forms. The transition-state analogue does delay inactivation on exposure to oxygen, because it locks aconitase into the transition-state configuration.

The large energy of activation for the slow release of the nitro inhibitor from aconitase is within the range observed for protein conformational changes (15–25 kcal mol^{-1}; Cleland, 1977) and therefore is consistent with the release being a consequence of a conformational change. The activation energy of 19.3 kcal mol^{-1} for the enzymic reaction suggests that a similar conformational change may occur during catalysis to allow product release. It should be emphasized that the conformational change following nitroisocitrate binding, or taking place during catalysis, is that postulated by conventional transition-state theory and, hence, distinct from the more unusual conformational change occurring in the activation process. The small values for the primary deuterium kinetic isotope effects with citrate and isocitrate (Thomson et al., 1966) and with methylisocitrate (J. V. Schloss, unpublished observation) are consistent with product release being rate limiting. Rose & O'Connell (1967) have also proposed that a conformational change occurs during the catalytic cycle, in order to explain the conversion of [3-^{3}H]isocitrate to [2-^{3}H]citrate without loss of tritium. They suggested that the exchange of the abstracted proton was limited by hydrogen bonding, so that its release to the medium would depend on the rupture of the hydrogen bond, caused or accompanied by a slow conformational change.

Scheme I is based on the evidence summarized above that in one transient state of the enzyme the Fe–S cluster is fully reduced but catalytic activity has not yet appeared to a major extent (e.g., Figure 1). Conversely, there is a transient state in which the cluster is already reoxidized, but catalytic activity is retained. Moreover, repeated cycling of the enzyme through two cycles of coulometric or chemical reduction and reoxi-

dation caused partial loss of the absorbance and EPR signal of the cluster, which was not quantitatively paralleled by loss of labile sulfide or of activity. Thus, Scheme I is minimal in that it does not take account of the growing evidence for the existence of aconitase in other states, which are either endowed with varying levels of catalytic activity or can be reduced to an active state. Recent Mossbauer experiments also gave clear indication of the presence of ~30% of a paramagnetic species of the reduced ferredoxin type in the dithionite-reduced enzyme, suggesting that the structure of this protein can accommodate Fe–S clusters other than the [3Fe–3S] type, while retaining activity (H. Beinert, J.-L. Dreyer, M. C. Kennedy, E. Münck, T. A. Kent, and B. H. Huynh, unpublished experiments). Thus, much remains to be done to characterize the various possible structures of this extraordinarily complex enzyme. The present study does establish, however, that the Fe–S cluster of aconitase, unlike these clusters in other enzymes, is not an electron donor or acceptor in catalysis, but rather, its redox state appears to control the ability of aconitase to undergo the conformational change which is part of the catalytic cycle.

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