The Role of Iron in the Activation-Inactivation of Aconitase*

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Methods are described for the convenient preparation of aconitase from beef heart mitochondria in its inactive [3Fe-4S] form and largely in its active [4Fe-4S] form. Inactive aconitase can be activated anerobically by various reducing agents without addition of iron. Under these conditions, maximally 70–80% of the activity attainable in the presence of added iron can be reached. It is concluded that during activation without added iron, [4Fe-4S] clusters are built from [3Fe-4S] clusters at the expense of a fraction of the 3Fe clusters present. This explains the ~75% maximal activation observed and concomitant loss of ~25% of total clusters as quantitated by EPR. Time course plots of aconitase activation appear to be second order but are not amenable to simple kinetic analysis because of the requirements of both reduction and Fe²⁺ for activation. Activation of aconitase with ⁵⁹Fe leads to rapid (minutes) incorporation of 1 iron atom/cluster, which on subsequent inactivation is readily lost again. With longer incubation times (hour), ⁵⁹Fe is found in more than a single site/cluster. It is concluded that, in analogy to cluster loss during activation in absence of added iron, the appearance of ⁵⁹Fe in more than one cluster site can be due to complete breakdown and rebuilding of clusters. However, exchange into intact clusters cannot be ruled out. Ferric iron can be bound nonspecifically to active and inactive aconitase but can be readily removed by chelating agents. Sulfide is not required for activation of aconitase in keeping with the proposal that inactive aconitase, as isolated, contains a [3Fe-4S] cluster. It is demonstrated that oxidation initiates the inactivation of aconitase with concomitant release of iron and formation of 3Fe clusters as determined by EPR.

The citric acid cycle enzyme, aconitase, had been known to be an Fe-S protein for only the past decade (1-3). Recent chemical analyses and extended x-ray absorption fine structure data suggest a [3Fe-4S]⁺⁻ structure for the oxidized 3Fe cluster (12). While Mossbauer spectroscopy played a decisive role in these studies, it was an important aspect in this development that our biochemical studies of the activation process and experiments by EPR and optical spectroscopy, as well as by tracer techniques, were fully in agreement with the interpretation suggested by the Mossbauer technique. It is the purpose of this series of papers to report the evidence obtained by means other than Mossbauer spectroscopy for the rebuilding of a [4Fe-4S] cluster from a [3Fe-4S] cluster during activation of aconitase and relating cluster type and oxidation state to enzymatic activity. The first paper will mainly focus on iron incorporation and release by chemical approaches and the second one on characterization by spectroscopic means of the species of aconitase involved.

MATERIALS AND METHODS

Chemicals—Isocitrate dehydrogenase, NADH, and NADP(H) were purchased from Boehringer-Mannheim; DL-isocitrate, cis-aconitate, Hepes, 2-(N-morpholino)ethanesulfonic acid, nitritotriacetic acid, N-hydroxysuccinimimidialtriacetic acid, and formamide sulfonic acid were from Sigma; ferrous ethylenediammonium sulfate from Merck, Darmstadt; 2,3-dioxyxibetrol and N,N-bis(2-hydroxyethyl)glycine from Calbiochem-Behring; phenanthrolines from G. F. Smith Co., Columbus, OH; dithionite from Fisher; and 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine p,p'-disulfonic acid (ferrozine) from Aldrich. ⁶⁷FeCl₃ and ¹³C-labeled EDTA were obtained from Amersham. Saffarin O was the kind gift of Dr. R. H. Burris (University of Wisconsin) and desferrioxamine (Desferal) of Dr. C. A. Brownley, Jr., of Ciba Pharmaceutical Co. Aequosil 1 from New England Nuclear was used for scintillation counting.

Anaerobic Techniques—Aconitase was routinely activated anerobically in small vessels, allowing activation in a total volume of 50 μl or more as required. These vessels had a small side arm and a 10/18 ground joint for connection to a line providing vacuum or argon purified through an Oxisorb cartridge (Messer-Griesheim). The main part of the vessel was filled with 50 μl Tris-CI (pH 8) or 0.1 M Hepes (pH 7.5), 5 mM dithiothreitol, and the enzyme; the side arm was purged with inert gas.

The abbreviations used are: Hepes, N-2-hydroxyethylpiperazine-N'·2-ethanesulfonic acid; FAS, formamide sulfonic acid; NTA, nitritotriacetic acid; HEDTA, N-hydroxysuccinimidialtriacetic acid; bicine, N,N-bis(2-hydroxyethyl)glycine.

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received 5 μl of a solution of ferrous ethylenediammonium sulfate in HCl at pH 2.0 (20 ml recently in 10 mM 2-(N-morpholino)ethanesulfonic acid (pH 5.5) for 50–100 μl total volume. After repeated evacuation and filling of the vessel with argon, the Fe solution was flushed into the main compartment with the bulk of the solution. A number of variants of this procedure, using other additions, activating agents, or other volumes of reagents, were employed as required for either method. Where used, formic acid (0.1 M) whatman p-83,000 was assumed (4). Sulfane sulfur, S⁺, was determined according to Petering et al. (16); however, complete absorption curves of the reaction mixtures after incubation were plotted and corrections were made for absorption of ferricyanide and Prussian blue, which are formed as by products in variable amounts. This introduces some uncertainty in the results. S⁺ was also determined according to Sakurai et al. (17) which appeared to us to be the quantitatively more reliable method.

Enzyme activities were assayed either by the coupled assay of Rose and O’Connell (18), in which NADP reduction is measured, or by following formation of aconitate from isocitrate (19). The latter assay (UV assay) was performed at 20°C in 90 mM Tris-Cl (pH 8.0) containing 20 mM DL-trisodium isocitrate. An extinction coefficient for cis-aconitate of 5.6 mM⁻¹ at 240 nm was used. Under these conditions, measured rates of 1800 μM/min⁻¹ were obtained, practically 2.8 times higher than those obtained in the coupled assay at 25°C. Unless otherwise noted, all specific activities will refer to the UV assay. EPR spectroscopy was carried out as described previously (20).

Preparation of Aconitase—Aconitase was purified from beef heart mitochondria using two procedures. The first method is a modification of a previously published procedure (21). The modifications allowed higher yields and shorter preparation times of inactive enzyme. The starting material was fresh mitochondrial paste (200–300 g wet weight) prepared by the procedure of Crane et al. (22) from slaughterhouse beef hearts. Tris-HCl (pH 7.4 at 20°C), 10 mM, containing 1 mM diethiothreitol was used in the beginning steps and shall be referred to as Tris buffer. All operations were performed at 0–4°C. The enzyme was released from mitochondria either by disruption for 1 min in a Waring Blender at 4°C or by sonication at 90 watts power for five 1-min intervals. In the former method the paste was suspended in 2 volumes of Tris buffer. In the latter method the mitochondria were washed twice in Tris buffer containing 0.25 M sucrose and suspended in 2 volumes of the same buffer (containing sucrose). The suspension was frozen at −10°C in 150–200-ml aliquots. This enzyme can be stored for several months with a significant decrease in yields. The broken mitochondria were then centrifuged for 80 min at 200,000 × g and the supernatant was decanted. This solution was applied to a column (5 × 30 cm) of Sigma DEAE-cellulose coupled to a column (5 × 15 cm) of Whatman CM52 cellulose, both equilibrated with Tris buffer. Under these conditions the major part of the aconitase activity remained on the CM52 column and after disconnecting the DEAE column could be eluted in a small volume with 50 mM Tris-HCl (pH 7.4). The eluate was then concentrated to 10–20 ml in an Amicon ultrafiltration cell using a PM-30 membrane and desalted into 8 mM potassium phosphate (pH 7.4) either by dialysis or gel filtration on Sephadex G-25. The final purification step was performed on CM-Sephadex C-50 (2.5 × 40 cm) using a linear gradient produced by 500 ml each of 8 mM and 25 mM potassium phosphate (pH 7.4). The purified enzyme (>95% purity as determined by sodium dodecyl sulfate-polyacrylamide electrophoresis) was then concentrated as described above to 20–100 mg/ml and stored in liquid nitrogen as 20–40-μl pellets. The yield of aconitase from 250 g of paste typically is 100–150 mg. Using the assay conditions of Rose and O’Connell (18), specific activities were 10–12 units/mg.

The second method combined the above purification procedure with the affinity elution method of Davies and Scopes (23) to yield 90% active aconitase. The initial steps are the same as described above in the first method except that storage of the frozen mitochondria was limited to a few weeks and the starting material was 50–70 g of mitochondrial paste. The supernatant from centrifugation was loaded on a column (5 × 10 cm) of Sigma DEAE-cellulose (5 × 15 cm) coupled to a column (5 × 8 cm) of Whatman CM52 cellulose, both equilibrated with 20 mM Hepes-KOH (pH 7.5). When all of the aconitase activity had passed through the DEAE-cellulose, that column was disconnected and the CM52 was washed with buffer until the remaining nonbinding protein was removed. The enzyme was then eluted with 20 mM Hepes-KOH (pH 7.5) containing 0.5 mM cis-aconitate. The eluate was concentrated and stored as described above. To minimize the oxidative inactivation of the enzyme, the time between loading the columns and elution with cis-aconitate should be 2–3 h. (The presence of substrate protects against oxidative inactivation.) The yield of aconitase (80% pure as determined by sodium dodecyl sulfate-polyacrylamide electrophoresis) from 60 g of paste is about 60 mg. This material has an unactivated activity of 9–11 units/mg as assayed by the procedure of Rose and O’Connell (18). In the following, “activated aconitase” will refer to enzyme obtained from the first method and then activated as described under “Materials and Methods,” while “active aconitase” will refer to enzyme obtained by the second method. Excess reagents were removed from aconitase when necessary by passing a 50–100-μl aliquot of the mixture through a Sephadex G-50 (fine) column (0.3 × 19 cm) or by centrifugation using the procedure outlined by Penefsky (24).

RESULTS AND DISCUSSION

Activation of Aconitase—The activation of inactive aconitase obtained on purification has been studied previously in several laboratories (6–8, 25). The requirements for agents such as cysteine or ascorbate and ferrous iron has been emphasized. Although all these compounds are reducing agents, no efforts were made to exclude oxygen and then to investigate the effect of reducing agents as such in the absence of iron. It became rapidly apparent in our studies that the activation process could not be adequately analyzed unless aerobic conditions were established. This is clearly illustrated in Fig. 1 where the aerobic activation with cysteine and Fe⁺⁺ and subsequent loss of activity on standing is monitored by assays on a 0.1- and 0.9-ml aliquot of the same initial activation medium. The aliquots were contained in test tubes of the same dimensions. Obviously the volume to surface ratio determines the rate of decline of activity; careful sampling from the bottom of the tube containing the larger sample actually showed no decline for some time. Fig. 2 shows the effect of Fe⁺⁺ concentration on aerobic activation with dithiothreitol and Fe⁺⁺. Assays were conducted aerobically because substrate protects the enzyme from inactivation. (see below) and transfer of activated enzyme samples from the aerobic activation medium to the assay medium in a few seconds does not lead to observable inactivation. It may be seen that when activation is carried out anaerobically, the sigmoidal time course reported by previous investigators (6, 7).
aconitase or aconitase loaded with iron by incubation with activated with FAS to the same specific activity as untreated. The ingredients of these media then showed that the levels of chelators as outlined in the subsequent section, could be considered whether the activity developed on addition of reducing agents without iron could be due to trace iron present in reducing agents. The most effective activation, irrespective of the time required, with some information on other conditions included. It can be seen that activities of 60–80% of the maximal reached in the presence of added iron can be achieved by reducing agents alone or combinations of reducing agents. The most effective activation, i.e. maximal activity in the shortest time, is observed with reducing agents, preferably a thiol, and iron. Addition of iron alone leads to maximal activity only after long incubation with an excess of iron. Because iron is a ubiquitous contaminant, we had to consider whether the activity developed on addition of reducing agents without iron could be due to trace iron present in the activation medium. Chemicals of the highest purity available were therefore chosen and the glassware used was treated to remove contaminating iron (27). Iron determinations on the ingredients of these media then showed that the levels of iron were such that, according to the data of Fig. 2, one would have expected minimal initial activities. A series of experiments was carried out on activation of aconitase with FAS as reductant, because we could not detect iron in this compound. We found that aconitase freed of spurious iron by treatment with chelators as outlined in the subsequent section, could be activated with FAS to the same specific activity as unreduced aconitase or aconitase loaded with iron by incubation with the Fe⁺⁺-sucrose complex (28) and then desalted. We conclude from these observations that spurious, nonspecifically bound iron does not, at least under these conditions, serve as a significant source of iron during activation. Manganese (Mn⁺⁺) may also be bound, but neither contributes to nor inhibits activation. All of these observations, viz. on the reagents that are absolutely required or merely helpful for activation, on the maximal activity achievable and on the time course of activation, point to three separate effects coming into play: reduction as an absolute requirement and additions of iron and thiol as helpful, but not absolutely required. While effects of these reagents on the time course of activation can be more readily rationalized, it was initially difficult to understand the observation that maximal activity could only be realized when iron was added. Both effects, namely that of thiols as well as of iron, can now be understood in terms of properties of Fe-S clusters. As documented by spectroscopic methods (12), the Fe-S cluster of aconitase may, under certain conditions, be relatively labile, thus making the enzyme itself the most likely source of iron. Seen in this light it is obvious that without added iron at best 75% of maximal activity can be reached if the active enzyme requires a [4Fe-4S] cluster, whereas the inactive enzyme only possesses a 3Fe cluster.

These relationships are documented in the experiments of Fig. 3. Here we make use of a technique more extensively used and also described in the accompanying paper (29), namely that of determining total cluster content of a solution of active aconitase by stoichiometric reoxidation with ferricyanide and quantitative determination by EPR spectroscopy. It is shown in this figure that aconitase activated in the absence of iron, with dithiothreitol and safranin O as oxidation-reduction mediator, develops maximally only 78% of the activity of enzyme activated in the presence of iron; and this activity corresponds, within error, to the content of 3Fe cluster found by EPR, viz. 75% of maximal. When aconitase had been activated by a reducing agent in the absence of iron and iron was added afterwards, there was always some increase in activity. This increase was variable and was inversely related to the activity reached with reductant alone but the

**TABLE I**

| Reagent added | Maximum activity achieved (%) |
|---------------|-------------------------------|
| Fe⁺⁺, DTT⁺⁺ | 100 h |
| Fe⁺⁺ | 100 0.5 h |
| DTT⁺⁺ | 75–80 5 h |
| DTT, safranin O⁺⁺ | 60–70 1.0 h |
| FAS⁺⁺ | 55–75 0.5 h |
| FAS, DTT | 60–80 0.5 h |
| Na₂S₂O₄ | 60–65 0.5 h |
| Ascorbate | 30–40 0.5 h |
| Ascorbate, DTT | 60–70 0.5 h |
| NADH (NADPH)⁺⁺ | 20–40 1.0 h |

**Fig. 2.** Time course for anaerobic activation of aconitase with iron concentrations of 10 μM (●), 25 μM (○), 100 μM (▲), and 500 μM (□). A modified double-necked activation vessel was used to allow sampling of the incubation mixture under argon pressure through a narrow bore stopcock. At time 0, iron solutions were mixed with 0.45 mg of inactive aconitase in 75 mM Hepes (pH 7.5) containing 5 mM dithiothreitol in a final volume of 0.2 ml. Aconitase activity was determined from 5-μl aliquots removed at various times.
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activity attainable, when both iron and reductant were initially present, was never reached (cf. Experiments 4–6 of Table II).

It is in line with these observations and the explanation suggested above that activation in the absence of added iron is always slow, i.e. the complete destruction of some and rebuilding of other clusters requires time; and it seems that in the process thiol, when added, will provide a medium favorable for transfer of iron between sites, as has been shown in chemical model studies (30).

The effect of substrate on the activation is complicated by the fact that substrate is bound at the active site and thus apparently stabilizes the labile iron atom of the [4Fe-4S] form (see below) but may also obstruct insertion of iron into the 3Fe form; and, in addition, citrate is a chelator of iron which will lower the oxidation-reduction potential so that iron becomes a better reductant (31). Fig. 4 illustrates this complexity. Activation in the presence of citrate is clearly more efficient than in its absence; however, at higher levels of citrate, iron seems to be prevented from entering the vacant cluster site through chelation by citrate and/or obstruction of access to the site by citrate.

Effects of Chelators—Another approach to verify or disprove a requirement for iron was the use of chelating agents. We found that the stability of the enzyme toward chelators depends critically on the state of the enzyme and the medium in which it is dissolved. Ferric or ferrous chelating agents, such as EDTA, NTA, desferrioxamine, and ferrozine, when incubated with the inactive enzyme and then removed again by gel exclusion chromatography, have little effect on the catalytic activity that can be generated by subsequent activation of the enzyme. However, when excess ferrozine, NTA, or EDTA is present in the activation mixture, there is little or no activation. When samples from the activation mixtures containing these chelators are examined by EPR spectroscopy, it becomes clear that the chelators neither interfere with reduction of the 3Fe clusters nor do they destroy the clusters, since on reoxidation by air or ferricyanide the original EPR signals were almost quantitatively recovered. Also by adding iron or even zinc equimolar to the chelator present or by removing the chelators by gel exclusion and then adding the

| Experiment | Aconitase | Dithiothreitol | Formamidine sulfonic acid | Incubation time | Aconitase activity | % of maximal | ng atoms/nmol | ng atoms/nmol |
|------------|-----------|----------------|--------------------------|----------------|-------------------|--------------|---------------|---------------|
| 1          | 148 µM    | 87 mM          | 2.5                      | 0               | 0.083             | 26           | 0.21          |               |
| 2          | 38 µM     | 170 mM         | 5.0                      | 0               | 0.083             | 100          | 0.90          |               |
| 3          | 38 µM     | 500 mM         | 5.0                      | 0               | 0.50              | 100          | 1.50          |               |
| 4          | 38 µM     | 170 mM         | +                        | (0.083)         | 0.50              | 75           | 0.73          |               |
| 5          | 38 µM     | 170 mM         | +                        | (0.50)          | 0.50              | 83           | 0.30          |               |
| 6          | 38 µM     | 170 mM         | +                        | (0.50)          | 0.50              | 70           | 0.70          |               |
| 7          | 230 µM    | 500 mM         | 5.0                      | 0.5             | 100               | 1.0          | 0.96          |               |
| 8          | 127 µM    | 714 mM         | 3.6                      | 48              | 100               | 1.6          | 1.0           |               |
| 9          | 279 µM    | 1,450 mM       | 7.1                      | 42              | 100               | 4.3          | 1.1           |               |

* The cluster concentration was estimated from chemical analysis with ferrozine (cf. Ref. 8) (one third of total iron) except for the samples in the last two rows, where cluster concentration was determined by EPR.

† By subsequent incubation with Desferal and nitrolotriacetic acid for 4 h at 0–2 °C and desalting. These samples had no activity after treatment.

‡ The sample was first incubated with formamidine sulfonic acid only for 0.5 h, then with iron (lower number).
usual activating quantities of iron back to the enzyme, normal activation can be achieved. Obviously, then, unless there is an excess of iron present, the chelators prevent activation by interfering iron liberated from decaying clusters which then becomes unavailable for building up \([\text{4Fe}-\text{4S}]\) clusters as they are required for enzyme activity.

The activated enzyme, after separation from the activating agents, is little affected by the mentioned chelators except on long incubation. Thus, for example, after 5 h aerobically at 0 °C, 76% of the activity remained with 0.65 mM ferrozine and 44% with 1 mM EDTA as compared to controls without chelator. As some of the chelators used are carboxylates, as are the substrates of aconitase, we ascertained that they did not inhibit aconitase activity in assays, as opposed to activation. When the coupled assay is used, care has to be taken that an excess of Mg\(^{2+}\) over chelator is present. We also tested by the use of \(^{14}C\)-labeled EDTA whether EDTA may be bound to aconitase. EDTA was readily removed from inactive or active aconitase by gel permeation chromatography. It was also confirmed by the Hummel-Dreyer method (32) that EDTA was not bound to inactive aconitase. The observations that chelators of the EDTA type are not bound but will effectively remove contaminating iron, provide a useful method for freeing the enzyme from non-cluster iron when the behavior of cluster iron is to be studied. This technique will be used extensively in the subsequent section.

Fig. 5 shows the relationship of the Fe/EDTA ratio and enzyme activity under reducing conditions. For this experiment the enzyme, dithiothreitol and EDTA concentrations are kept constant and only the Fe\(^{2+}\) concentration is varied. At low concentrations of Fe\(^{2+}\) (25 \(\mu\)M), aconitase will be fully active in 30 min (see Fig. 2) but shows only 1% of this activity in the presence of 100 \(\mu\)M EDTA. As the Fe\(^{2+}\) concentration is increased, aconitase can compete effectively with EDTA for Fe\(^{2+}\), as measured by the increase in enzymatic activity. The association constant for aconitase and Fe\(^{2+}\), i.e. the formation constant of 4Fe clusters in aconitase from 3Fe clusters and iron under reducing conditions, can be estimated from the competition with EDTA. 2 Values for log \(K\) of [4Fe-

\[
K_{\text{Fe(EDTA)}} = \frac{[\text{4Fe-4S}]}{[\text{FeEDTA}][\text{3Fe-4S}]} = \frac{K_{\text{Fe(4S)}}}{K_{\text{EDTA}}} \tag{1}
\]

where

\[
K_{\text{Fe(EDTA)}} = \frac{[\text{EDTA}][\text{4Fe-4S}]}{[\text{FeEDTA}][\text{3Fe-4S}]} = \frac{K_{\text{Fe(4S)}}}{K_{\text{EDTA}}} \tag{2}
\]

Using procedures outlined by Pecoraro et al. (33), [EDTA] and [FeEDTA] of Equation 2 are calculated from known protonation and hydrolysis constants of EDTA and Fe(EDTA), respectively (34). The activity of aconitase is used to determine the [4Fe-4S]/[3Fe-4S] ratio (see accompanying paper (39)). The log \(K_{\text{Fe(EDTA)}}\) has a known value of 14.32 (34). The \(K_{\text{Fe(4S)}}\) can then be calculated from data where the total EDTA exceeds total iron. Preliminary data from three other chelators (bicine, log \(K_{\text{bic}}\) = 4.3; NTA, log \(K_{\text{NTA}}\) = 8.3; and HEDTA, log \(K_{\text{HEDTA}}\) = 12.3 (34)) were obtained. The extent of activation achieved under similar conditions with each of the four chelators is related to the amount of free Fe\(^{2+}\) available (i.e. bicine > NTA > HEDTA > EDTA). To measure the formation constant of active aconitase at the chelator-enzyme ratio used (25:1), the \(K_{\text{Fe(EDTA)}}\) of the chelator must be greater than that of aconitase. In the presence of bicine or NTA full activation can be achieved at low Fe concentrations. This places a lower limit on log \(K_{\text{Fe(EDTA)}}\) of ~7 with bicine and ~9 with NTA. HEDTA gave formation constant values lower than those obtained with EDTA. While time course studies indicated that the highest activity in the presence of EDTA occurred within a few minutes, a slow increase in activity is observed over several hours with HEDTA. The discrepancy between the data of EDTA and HEDTA may be caused by an inability to attain a true equilibrium. Inactivation of aconitase (i.e. the loss of the added Fe) appears to be initiated by oxidation (see “Results”). Thus under reducing conditions, where little inactivation is likely to occur, a true equilibrium between Fe-chelator and aconitase may not be reached. Therefore, the formation constants obtained for iron activation of aconitase should be viewed as estimates only.

**Fig. 5. Effect of iron to EDTA ratio on the activation of aconitase.** Aconitase (0.5 mg) was anaerobically activated in 80 mM Hepes (pH 7.5) containing 5 mM dithiothreitol, 100 \(\mu\)M EDTA, and varying iron concentrations. The samples were incubated for 30 min at room temperature before determining activity.

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2 The formation constant of active [4Fe-4S] aconitase from Fe\(^{3+}\) and inactive [3Fe-4S] aconitase is estimated from the competition reaction:

\[
\text{Fe}^{2+} + [\text{EDTA}] + [\text{4Fe-4S}] \rightleftharpoons [\text{FeEDTA}] + [\text{3Fe-4S}] \tag{1}
\]

where

\[
K_{\text{comp}} = \frac{[\text{FeEDTA}][\text{3Fe-4S}]}{[\text{Fe}^{2+}][\text{EDTA}][\text{4Fe-4S}]} = \frac{K_{\text{Fe(4S)}}}{K_{\text{EDTA}}} \tag{2}
\]

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plus NTA as carrier or EDTA followed by passage through a Sephadex G-50 column shows that >95% of this iron is readily removed. The $^{59}$Fe taken up during the activation process, however, is partly retained on gel exclusion chromatography or on treatment with chelators. The binding of ferric and ferrous $^{59}$Fe by the active aconitase preparation, which largely contains [4Fe-4S] clusters (see "Materials and Methods"), was tested. While the enzyme bound 2-3 mol/mol of ferric ion, no significant binding of ferrous ion was observed. This indicates the presence of nonspecific ferric ion binding sites on the protein.

Table II presents experiments in which the aconitase activity reached under various conditions of activation is compared to the uptake and also in Experiments 7-9 the retention of $^{59}$Fe added during activation. In Experiments 1-4 a correlation between iron uptake and activity developed becomes evident. Full activity is only reached when approximately 1 iron atom/cluster is taken up. A comparison of Experiments 5 and 6 shows that similar activity can be reached when iron is offered for 5 min or for 30 min after reduction with FAS, but incorporation of the added $^{59}$Fe is more extensive on long incubation (30 min) with iron. Experiments 7-9 show that on activation at 4-6 °C for about 2 days up to 4 iron atoms/cluster may be taken up but that only one iron atom is readily removed on exposure to NTA and desferal under aerobic conditions. More elaborate experiments are shown in Table III. Three experiments are presented, in which the enzyme was activated anaerobically by incubation with dithiothreitol and $^{59}$Fe, freed of low molecular mass reactants and then exposed aerobically to the iron chelators NTA and desferal. In the first experiment the enzyme was then once more activated with dithiothreitol only, without extraneous iron, to show its viability after the treatment with the chelators and to recheck for retention of iron after renewed exposure to chelators after the second activation. Radioactivity and Fe-S cluster concentration, according to the EPR signal at $g = 2.01$, were followed throughout the procedure and at some points iron was determined by chemical analysis and enzymatic activity by assay. From a comparison of Experiments 7 and 8 with 9 (Table II), it can be seen that on long exposure under activating conditions up to 4 iron atoms are initially taken up per cluster, of which one is removed on aerobic exposure to the iron chelators, while on very short (10 min) activation (Table III) only 0.2 Fe/cluster is retained after the aerobic chelator treatment. It can also be seen in Experiment 1 (Table III) that after the first incubation with chelators, about 3 Fe/cluster are retained in the 3Fe cluster formed on exposure to oxygen.

We explain the results of the experiments given in Tables II and III in the following way. On activation with $^{59}$Fe, the isotope is taken up into one specific position in the newly formed [4Fe-4S] cluster of the active enzyme. On oxidation by air, this iron is again specifically lost from that site. On continued exposure, however, to activating conditions a dynamic situation is likely to exist, with clusters being partly or completely disassembled and reassembled—a process analogous to that which allows for activation in the absence of added iron—in the course of which eventually all four sites of the cluster may become labeled, not just the site of the primary entry of iron into a pre-existing 3Fe cluster. When the protein is then exposed to oxygen in the presence of chelators, iron is only removed from the site of primary entry but the iron in the other sites is retained. This then becomes evident in the drop of the amount of iron retained from about 4/cluster to 3/cluster. Since Mössbauer spectroscopy has shown that during activation $^{59}$Fe is initially incorporated only into a single site/cluster and can be readily removed again by exchange with $^{56}$Fe (12), we identify this site as that which largely lost its label in Experiments 7-9 of Table II and Experiments 1 and 2 of Table III. The fact that during long incubation times under activating conditions aconitase can be activated to 75% of original activity without addition of iron, furnishes support for the idea that in these time intervals complete breakdown and reutilization of the liberated iron are an essential feature of the mechanism of incorporation.

Experiment 2 of Table III was carried out at pH 10 because

### Table III

**Balance of $^{59}$Fe uptake and removal, total iron, and Fe-S cluster concentration during activation-inactivation of aconitase**

| Experiment | Treatment | Aconitase | Dithiothreitol | Nitrilotriacetic acid (2 mM) | Desferal (50 mM) | Aerobic | Time | Temperature | $^{59}$Fe | $^{56}$Fe | Fe-S cluster | $^{59}$Fe/cluster |
|------------|-----------|-----------|----------------|-----------------------------|----------------|--------|------|-------------|--------|--------|---------------|----------------|
| 1          | Incubate  | 60 (140)  | 7.1            |                            |                |        | 42   | 4-6         | 1.43 (200) | 80,934 (200) | 28.3 | 4.3 |
| Desalt     | 75        |           |                |                            |                |        | 0.56 (64.5) | 30,000 (74.3) | 17.1 | 3.2 |
| Incubate   | 5         | 5         | +              |                            |                |        | 5    | 0-2         | 7,619 (18.8) | 5.8    | 3.2 |
| Desal      | 42        |           |                |                            |                |        | 3,979 (9.8) | 1,100 (2.7) | 1.1    | 2.5 |
| Incubate   | 10 (100)  | 5.0*      |                |                            |                |        | 0.5  | 18          | 1,100 (2.7) | 1.1    | 2.5 |
| Desal      | 40        |           |                |                            |                |        | 3,979 (9.8) | 1,100 (2.7) | 1.1    | 2.5 |
| Incubate   | 40        |           |                |                            |                |        | 3.5  | 0-2         | 1,100 (2.7) | 1.1    | 2.5 |
| Desal      | 20        |           |                |                            |                |        | 3,979 (9.8) | 1,100 (2.7) | 1.1    | 2.5 |
| 2          | Incubate  | 30 (150)  | 3.3            |                            |                |        | 44   | 4-6         | 0.67 (100) | 40,467 (100) | 14.1 | 4.4 |
| Desal      | 75        |           |                |                            |                |        | 0.40 (30) | 12,500 (30.9) | 7.0    | 4.4 |
| Incubate   | 5         | 5         | +              |                            |                |        | 4    | 0-2         | 0.24 (9.6) | 2,981 (7.36) | 2.6  | 2.8 |
| Desal      | 40        |           |                |                            |                |        | 2,981 (7.36) | 1,100 (2.7) | 1.1    | 2.5 |
| 3          | Incubate  | 30 (150)  | 3.3            |                            |                |        | 0.17 | 18          | 0.67 (100) | 40,467 (100) | 14.1 | 4.4 |
| Desal      | 50        |           |                |                            |                |        | 5,652 (14) | 7.5  | 1.9 |
| Incubate   | 5         | 5         | +              |                            |                |        | 4    | 0-2         | 0.36 (10.8) | 219 (0.5)  | 2.5  | 0.2 |
| Desal      | 30        |           |                |                            |                |        | 219 (0.5) | 1,100 (2.7) | 1.1    | 2.5 |

* By chemical analysis with ferrozine (cf. Ref. 8).
* Experiment 1 in Tris (pH 7.5); 2 in Tris-Hepes (pH 7.5); 3 in glycine-NaOH (pH 10).
* Formamidine sulfonic acid was added as reductant.
assays of enzyme activity and EPR spectroscopy had shown that cluster breakdown is accelerated at this pH. One would therefore have expected a more rapid equilibration of $^{59}$Fe. We failed to see such an effect probably because of the long incubation times chosen, which may have allowed complete equilibration even at pH 7.5.

We have no reason to doubt the validity of the experiments presented in Tables II and III for the conditions specified there. However, in subsequent experiments it was found that the incorporation of $^{59}$Fe into sites other than that of primary entry can be variable, depending on the experimental conditions. These conditions include concentration of the enzyme and $^{59}$Fe, pH, time, and presence or absence of dithiothreitol or chelator.

Release of Iron from Aconitase—In the previous section experiments were described in which Fe was incorporated into the inactive 3Fe form of aconitase, which is obtained on purification. We have sought ways to conduct the reverse reaction, namely iron removal, under controlled conditions so that it could be studied in more detail. This reaction, of course, occurs spontaneously on exposure of the active [4Fe-4S] form to air. However, as during the activation, the participation of a gaseous reactant, O$_2$, complicates the situation unnecessarily. We found a convenient way of controlling the decay of the [4Fe-4S] cluster in the use of persulfate as a relatively mild oxidant and appropriate levels of substrate to slow the decay to a convenient rate (persulfate inactivates aconitase cluster (EPR) were followed with time. Fig. 6 shows an experiment of this series conducted on the active aconitase preparation with 33 $\mu$M citrate present. Molarity of 3Fe cluster is plotted on the ordinate on the same scale as molarity of iron released and bound by ferrozine. It can be seen from this plot that initially 3Fe cluster formation and appearance of iron almost coincide, indicating that 1 iron atom is extruded from the active enzyme to form 1 3Fe cluster/molecule of active enzyme. In this period, 15–20 min in this experiment, most of the activity is lost. As time progresses, however, more iron appears than can be accounted for by release of a single iron atom/cluster, and 3Fe cluster formation falls beyond the maximum possible according to the iron and original cluster content of the enzyme used, viz. 9 $\mu$M. This clearly indicates that as the oxidative degradation proceeds, first [4Fe-4S] clusters are converted to the inactive 3Fe form; but more slowly total destruction of clusters also occurs which, after all activity is lost, continues for hours. If specific activity is plotted against Fe released (not shown), there is an initial linear correlation between loss of activity and loss of Fe. Extrapolation to the zero intercept occurs at 8.2 $\mu$M Fe which is 22% of the total Fe originally present in the enzyme. This is quite close to the expected 25% Fe released for the conversion of a 4Fe to a 3Fe cluster. The maximal iron that could be released from the sample after sodium dodecyl sulfate treatment amounted to 37.6 $\mu$M. This agrees well with 35 $\mu$M expected from the initial cluster concentration. There is also a curve shown in Fig. 6 (x) which indicates considerably slower loss of activity. In the corresponding experiment, 5.9 mM citrate was present, showing the very effective protection of aconitase by substrate. The dotted curve (x) refers to the decline of activity in an experiment in which ferrozine was omitted. The fact that this curve coincides with the ordinate of the corresponding curve obtained in the presence of ferrozine (o) shows that the presence of the iron chelator has no significant effect on the conversion of [4Fe-4S] to 3Fe clusters. Variation of the ferrozine concentration, similarly, made no difference. The oxidative attack appears to be the primary effect in this cluster conversion. Fig. 7 shows an experiment from which we can clearly recognize the protective effect of substrate in comparison to Fig. 6. In this experiment activated aconitase was used with no substrate present. The decline of activity is precipitous, so is 3Fe cluster formation; and the maximal level of 3Fe cluster formed is close to the level that would be expected if the 3Fe clusters were stable (3.5 $\mu$M). Iron release again proceeds at a slower rate after loss of activity, indicating eventual complete breakdown of 3Fe clusters which were formed within the first 3 min from [4Fe-4S] clusters. The Source of Labile S$^{2-}$ in Cluster Interconversions—While we can generate maximal activity by adding iron and a reduc tant to unactivated aconitase, a need for or effect of adding S$^{2-}$ has never been observed. As long as aconitase was still considered to contain a [3Fe-3S] cluster (5, 12), it was therefore necessary to explain the origin of the S$^{2-}$ required in the
conversion of 3Fe to [4Fe-4S] clusters. In analogy to work by Petering et al. (16), we analyzed aconitase samples for S~. The amounts found were variable but amounted to about 1 S~/1.5 Fe. However, we found no difference between aconitase preparations of high and poor activity or of fresh samples and samples sitting in air at room temperature for hours. Our failure to see such a difference suggests that we may have generated most of the detected S~ from the enzyme while it was incubated with cyanide at alkaline pH to produce the thiocyanate according to the method used.

On the other hand, we had been finding regularly somewhat more S~2- than iron in all preparations. It may be remembered that one of us (M. C. K.) had consistently found iron and S~2- at a ratio of 2:3 in preparations of pig heart aconitase (1, 35). Since the analytical method for labile S~2- relies on an empirical, not a stoichiometric, relationship between S~2- present and methylene blue formed (36), we were hesitant to attribute particular significance to the ratio of Fe/S~2- of 0.9 found with our preparations and analyses up to 1981. However, progress in devising a reproducible semimicro version of the standard procedure for S~2- analysis (37) has now made it possible to ascertain that the ratio of iron to labile S~2- is 0.73 ± 0.04, which led us to the conclusion that aconitase as isolated contains a [3Fe-4S] cluster rather than a [3Fe-3S] cluster (13).

CONCLUSIONS

To summarize the conclusions we draw from the experiments reported here in the light of the spectroscopic work reported previously. Activation of aconitase proceeds on reduction of its Fe-S cluster. It is not necessary to add iron. Under reducing conditions iron becomes available from a reorganization of clusters resulting in formation of [4Fe-4S] clusters at the expense of a fraction of the 3Fe clusters originally present. Because of this loss of clusters, only ~75% of the activity which would be achievable on addition of iron can be reached by reductant alone. This facilitates the cluster interconversion. On activation, in the presence of iron, one iron atom per 3Fe cluster is readily incorporated within a few minutes. Full activity is not reached unless 1 iron/cluster is incorporated. This iron atom is readily lost again on oxidation of the cluster. On longer exposure of aconitase to an activation medium containing iron, more iron atoms in the Fe-S cluster may be equilibrated with iron in the medium presumably via partial or complete breakdown and reformation of [4Fe-4S] clusters. Chelating agents for ferric and ferrous iron (not including phenanthroline) do not readily degrade 3Fe or [4Fe-4S] clusters. They will, however, prevent activation unless excess iron is present. Cluster degradation is initiated by oxidation, with [4Fe-4S] clusters rapidly forming 3Fe clusters and 3Fe clusters more slowly decaying further.

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