Molecular forms of aconitase and their interconversions

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Aconitase, as isolated from mammalian mitochondria by traditional methods, is virtually inactive and contains an oxidized $[3\text{Fe}-4\text{S}]^+$ cluster. The activation of the enzyme and attendant conformational change have been studied by monitoring the changes in activity, in tryptophan fluorescence, and in the electron paramagnetic resonance of the cluster on incubation with dithionite, with and without added Fe^{2+} . Restoration of the full activity is achieved with one electron per 3Fe cluster and at least 0.6g-atoms of Fe^{2+} per mol. The process involves building up of $[4\text{Fe}-4\text{S}]^{2+}$ clusters. Other metal ions do not substitute for Fe^{2+} . Reduction alone, in the absence of added Fe^{2+} , yields up to 70% of the maximum activity, but requires approx. 1.8 electrons of reductant per cluster. The results presented are consistent with the view that activation without added Fe^{2+} involves the destruction of some of the $[3\text{Fe}-4\text{S}]$ clusters and the incorporation of the Fe so liberated into other clusters to yield a tetranuclear one. In particular, the effect of EDTA and of other iron chelators in inhibiting activation by dithionite alone is in accord with this view, although recent magnetic-circular-dichroism studies do not support this interpretation. The rates of increase in activity and tryptophan fluorescence are the same when Fe^{2+} is present, but in its absence, activation is very much slower than the increase in fluorescence, suggesting that the protein conformational change triggered by reduction of the Fe-S clusters precedes the insertion of the iron. Consistent with this view is the observation that iron chelators inhibit activation by dithionite, but not the increase in fluorescence and, hence, the conformational change. The results are discussed in light of data in the literature on the forms of the cluster and its possible function in catalysis.

Recent investigations of aconitase have probed the structure of its unusual iron-sulphur cluster by physical techniques such as Mössbauer, e.p.r., e.x.a.f.s., resonance Raman and m.c.d. spectroscopy (Kent *et al.*, 1982; Emptage *et al.*, 1983a; Beinert *et al.*, 1983; Johnson *et al.*, 1983, 1984) and the transformations of the cluster underlying the activation-deactivation of the enzyme (Kennedy *et al.*, 1983; Emptage *et al.*, 1983a,b). Studies in this laboratory have concentrated on the changes in the protein during the activation-deactivation

cycle (Ramsay *et al.*, 1981; Ramsay, 1982). We can now interpret our data in light of the structures of the Fe-S cluster in different forms of the enzyme which have recently been established by Mössbauer and e.p.r. studies (Beinert *et al.*, 1983; Emptage *et al.*, 1983a; Kennedy *et al.*, 1983).

As isolated, ox heart aconitase contains 2.4–2.8g-atoms of non-haem Fe per molecule (83kDa) and an Fe/labile-S ratio of 0.66–0.74, compatible with a structure consisting of 3Fe and 4S (Beinert *et al.*, 1983; Johnson *et al.*, 1983). The activity of the form isolated depends on the speed of isolation and the degree of anaerobiosis maintained (Ruzicka & Beinert, 1978; Kennedy *et al.*, 1983; Singer & Ramsay, 1984). On anaerobic incubation with an excess of Fe^{2+} and a thiol, such as DTE, full activity is reached. Physical measurements show that the cluster in the enzyme thus activated

Abbreviations used: e.x.a.f.s., extended X-ray absorption fine structure; m.c.d., magnetic circular dichroism; DTE, dithioerythritol; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; SDS, sodium dodecyl sulphate.

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is of the oxidized ferredoxin type, $[4\text{Fe-4S}]^{2+}$ (Kent *et al.*, 1982; Emptage *et al.*, 1983a; Kennedy *et al.*, 1983), which is now believed to be the native, fully active form.

Partial but significant activation has also been observed, however, after anaerobic reduction with dithionite without an added source of Fe^{2+} (Ruzicka & Beinert, 1978; Kent *et al.*, 1982; Ramsay, 1982; Kennedy *et al.*, 1983), and maximal activation has been observed after coulometric reduction (Ramsay *et al.*, 1981). Although the latter has been explained by the demonstration (H. Beinert, personal communication) that Fe^{2+} was derived from the ferrocyanide which was present as mediator during electrochemical activation (Singer & Ramsay, 1984), external sources of iron were rigorously excluded in the experiments with dithionite. Kent *et al.* (1982) reported that such dithionite-activated preparations are e.p.r.-silent, although still paramagnetic, and that the predominant form of the cluster detected by Mössbauer spectroscopy is the reduced $[3\text{Fe-4S}]^0$ form, a finding we confirmed by m.c.d. studies (Johnson *et al.*, 1984).

Later, further Mössbauer studies revealed some $[4\text{Fe-4S}]^{2+}$ in dithionite-treated samples, roughly in proportion to the activity elicited, and suggested that the extra iron had been derived by cannibalization of some 3Fe clusters (Emptage *et al.*, 1983a). In our m.c.d. experiments (Johnson *et al.*, 1984), no 4Fe cluster was detected, although samples of the dithionite-treated preparations showed 66% of the maximal catalytic activity. Thus the question as to whether the $[4\text{Fe-4S}]^{2+}$ form of aconitase is the only one endowed with catalytic activity has not been unambiguously answered.

During reductive activation of the enzyme there is a lag between the change in the redox state of the Fe-S cluster, as monitored by absorbance changes, and the appearance of activity (Ramsay *et al.*, 1981). We interpreted this lag as evidence for a slow conformational change in the protein, which then led to the change in activity. Evidence for such a conformational change came from the finding that the increase in activity was accompanied by an increase in tryptophan fluorescence (Ramsay, 1982). On titration with dithionite the changes in the absorbance of the Fe-S cluster, in the fluorescence of the protein, and in catalytic activity, were closely correlated, but occurred at different rates. The absorbance change, representing the reduction of the cluster, was immediate, but the increases in activity and fluorescence required about 10 min at 15°C for completion. The present paper describes the events occurring during activation of the enzyme in terms of accrued knowledge of its Fe-S cluster.

Experimental

Enzyme and assays

Aconitase was prepared from ox heart mitochondria as described by Ruzicka & Beinert (1974), except that oxygen was excluded as much as was possible during the isolation procedure. The activity of aconitase was determined after activation by anaerobic incubation for 30 min at room temperature of 0.15–1 mg of protein in 0.5 ml of 25 mM-potassium phosphate, pH 7.4, with 0.5 mM- FeSO_4 and 0.5 mM-dithioerythritol. This method of activation was found to give more reliable results than that described by Gawron *et al.* (1974b). The specific activity of aconitase so activated was 9–13 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ in the coupled assay system of Rose & O'Connell (1967), but, as isolated, the activity varied from 15 to 50% of the maximum. Analytical assays have been described previously (Ramsay *et al.*, 1981). The preparations contained 2.4–2.8 g-atoms of Fe/molecule of 83 kDa molecular mass. All samples were subjected to aerobic centrifugal gel filtration (Penefsky, 1977) on columns of Sephadex G-50 (fine grade) in 25 mM-potassium phosphate, pH 7.4, to remove DTE and to oxidize the aconitase.

Activation and fluorescence

The activation of aconitase by anaerobic incubation at 15°C with dithionite was performed as described previously (Ramsay, 1981), except that additions of dithionite, Fe^{2+} or EDTA were mixed by shaking for 5 s. The fluorescence of the sample [1.5–2 mg of protein in 4 ml of 25 mM-potassium phosphate, pH 7.4, containing 5 mM-glucose, glucose oxidase (1 unit·ml⁻¹; Miles Laboratories) and catalase (24 units·ml⁻¹; Sigma Chemical Co.)] at 333 nm ($\lambda_{\text{ex.}} = 295 \text{ nm}$) was monitored continuously after the addition of dithionite. Samples for activity were removed under a flow of argon, but it is difficult to be certain of complete anaerobiosis during the 20 s required for transfer to the assay cuvette. Assays were performed aerobically in the experiments described. In other experiments, we found that, at high concentrations of dithionite ($>3 \text{ e}^-/\text{mol}$) and in the presence of Fe^{2+} , slightly lower activities were found in anaerobic assays than in aerobic, in agreement with Kennedy *et al.* (1983).

E.p.r.

Samples for e.p.r. spectroscopy were prepared as described above, but in 20 mM-Hepes, pH 7.4, at 8 mg of protein·ml⁻¹. Oxidized, inactive aconitase was compared with samples that had been treated with 2.2 or 16.6 e^- per 3Fe in the absence or presence of 2-fold excess of Fe^{2+} . Samples were monitored for 3Fe clusters at 12 K (microwave

power 1 mW, modulation amplitude 0.32 mT, microwave frequency 9.09 GHz) and for 4Fe clusters at 20 K (microwave power 20 mW, modulation amplitude 1.0 mT, microwave frequency 9.1 GHz).

Miscellaneous

All glassware was kept as iron-free as possible by cleaning it overnight in warm HNO_3 . Anaerobic cuvettes were washed with EDTA, then with HNO_3 . The buffer solution (25 mM-potassium phosphate, pH 7.4) was passed through a column (2 cm $\times$ 30 cm) of Chelex 100 chelating resin (Bio-Rad Laboratories) at 2 ml/h. Dithionite was kept in a desiccator and made up anaerobically in 5 mM- K_2HPO_4 . The concentration of the solution was determined by titration against 40 μM -riboflavin as described by Edmondson & Singer (1973), except that argon gas replaced helium.

Results

Effect of dithionite on catalytic activity and fluorescence

The time course of the activation of an aerobically oxidized sample of aconitase at 15°C by various amounts of dithionite is shown in Fig. 1. The maximal activity reached is clearly a function of the concentration of the reductant. The concentration of 3Fe clusters in the sample was 4.1 μM , but 3.16 μM -dithionite (supplying twice that concentration of electrons) gave incomplete activation, so it was clear that $1.54e^-$ per 3Fe cluster were insufficient for maximal activation. Note that the reduction of a $[3\text{Fe}-4\text{S}]^+$ cluster, which is predominantly present in the inactive enzyme, to $[3\text{Fe}-4\text{S}]^0$ requires only $1e^-$, as does its conversion into $[4\text{Fe}-4\text{S}]^{2+}$, if the extra Fe is supplied as ferrous iron.

Fig. 1 also demonstrates that the anaerobic addition of a 3-fold excess of Fe^{2+} after a plateau had been reached at each level of dithionite gave a rapid and significant increase in specific activity, but the increase produced was proportional to the activity attained with dithionite alone. This suggested that the additional activity might be due to modification of the already-reduced clusters by the added Fe^{2+} .

Fig. 2 shows titration curves for the increase in activity observed before and after incubation with Fe^{2+} and for the increment in tryptophan fluorescence. The inflection point represents about $1.8e^-$ per 3Fe cluster for the maximal activities and fluorescence reached. This corresponds to about 1.5 electrons per molecule of aconitase (83 kDa). The excess of dithionite previously thought to be required may have been due to traces of oxygen that had not been removed by the glucose oxidase

Fig. 1. Activation of aconitase after reduction by dithionite. Aconitase ($0.42 \text{ mg} \cdot \text{ml}^{-1}$; $5.1 \mu\text{M}$ based on protein and a molecular mass of 83 kDa or $4.1 \mu\text{M}$ based on Fe determination and assuming that all the Fe is in the form of 3Fe clusters) was incubated anaerobically at 15°C as described in the Experimental section. Portions of dithionite were added to separate samples to give final concentrations of 1.05–15.8 μM and samples taken from the incubation mixture were assayed for activity. When the activity reached a plateau, FeSO_4 (in anaerobic solution) was added, where indicated by the arrows, to give a final concentration of 12.5 μM . The activity reached after the addition of Fe^{2+} was constant for at least 15 min longer than shown. The specific activity of a control sample activated by anaerobic incubation with Fe^{2+} + DTE was $9.3 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ and the enzyme was 83% pure as judged by SDS/polyacrylamide-gel electrophoresis. The concentrations of dithionite in the cuvettes were: ●, 1.05 μM ($0.52e^-/3\text{Fe}$); ○, 3.16 μM ($1.54e^-/3\text{Fe}$); ■, 10.5 μM ($5.2e^-/3\text{Fe}$); and □, 15.8 μM ($7.8e^-/3\text{Fe}$).

system. In the early experiments, the sample was bubbled with unscrubbed argon, but the experiments reported here were performed under a positive pressure of argon that had been passed through a purifier cartridge (Labclear, Oakland, CA, U.S.A.).

Samples that had been reduced with 2.2 and 16.6 $e^-/3\text{Fe}$ cluster respectively without added Fe^{2+} were examined by e.p.r. spectroscopy, which can detect the oxidized, inactive $[3\text{Fe}-4\text{S}]^0$ form ($g = 2.01$) and the reduced $[4\text{Fe}-4\text{S}]^+$ form ($g = 1.94$), but not the fully active $[4\text{Fe}-4\text{S}]^{2+}$ form (Emptage *et al.*, 1983a). At the lower level of

Fig. 2. Changes in fluorescence and activity when aconitase is activated by different amounts of dithionite

The data come from an experiment that included the time courses shown in Fig. 1. The plateau activity in the time course, reached after addition of dithionite alone (○), after subsequent addition of $12.5 \mu\text{M-Fe}^{2+}$ (●), and the increase in fluorescence that accompanies the activation by dithionite (□) are shown as a function of the electrons from dithionite added per 3Fe (based on non-haem-iron analysis). The concentration of aconitase was $4.1 \mu\text{M}$ (on the basis of Fe analysis).

dithionite, approx. 1% of the signal typical of oxidized aconitase (centred at $g = 2.01$) was seen, and even less in the other sample. At the higher level of dithionite, a small signal (<5% of the clusters) was observed with g -values of 2.01, 1.82 and 1.75 (not shown), similar to the signal obtained for aconitase in the $[4\text{Fe-4S}]^+$ form in the presence of substrate (Emptage *et al.*, 1983a,b). It suggests that some substrate was present in our sample and that at least a small amount of $[4\text{Fe-4S}]^+$ cluster had been formed, but far less than would be necessary to explain 70% activity. There was no signal centred at $g = 1.94$, in contrast with the spectrum obtained in the presence of Fe^{2+} (see below).

When aconitase was titrated anaerobically with dithionite in the presence of $12.5 \mu\text{M-FeSO}_4$ (3.5-fold excess over the cluster concentration), materially different results were obtained (Fig. 3). Activity and fluorescence reached a maximum at one electron equivalent of dithionite per mol of 3Fe cluster. An e.p.r. signal at $g = 1.94$ typical of reduced ferredoxin was present in the sample reduced with $2.2e^-/3\text{Fe}$ cluster and very prominent in the one that had been treated with $16.6e^-/3\text{Fe}$ cluster in the presence of Fe^{2+} . (In both cases, presumably owing to the presence of a small amount of substrate, the small signal with g -values 2.01, 1.82, 1.75 was also present, at a level of less than 10% of the 1.94 signal in the $16.6e^-$ -

Fig. 3. Changes in fluorescence and activity when aconitase is activated by different amounts of dithionite in the presence of Fe^{2+}

The experiment was analogous to that in Fig. 2, except that FeSO_4 ($12.5 \mu\text{M}$) was added to the anaerobic cuvette containing aconitase ($0.36 \text{ mg} \cdot \text{ml}^{-1}$; $3.6 \mu\text{M}$ on the basis of Fe analysis) immediately before the addition of dithionite. The specific activity of a control sample activated by $\text{Fe}^{2+} + \text{DTE}$ was $8.4 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$. (The sample was 80% pure as judged by SDS/polyacrylamide-gel electrophoresis.)

reduced cluster.) These results agree with those of Kent *et al.* (1982) and Emptage *et al.* (1983a).

Effect of added Fe^{2+}

On comparing the total change in activity and fluorescence elicited by Fe^{2+} during reductive activation by excess dithionite ($10e^-$ equivalents/3Fe cluster), it was found that Fe^{2+} increased the maximal activity by 20–25%, but decreased the fluorescence by a similar amount (result not shown; see Singer & Ramsay, 1984). The significance of these differences will be discussed below. In contrast, the rate at which the changes in fluorescence and activity were attained were both increased by Fe^{2+} . The maximal effects of added Fe^{2+} on all these parameters was seen at 0.6 g-atom of $\text{Fe}^{2+}/3\text{Fe}$ cluster; above this ratio, no further effect of Fe^{2+} was noted: Since no adventitious iron could be detected in the chemicals used, it is not clear why less than a 1:1 ratio of $\text{Fe}^{2+}/3\text{Fe}$ cluster was required for maximal effect. It should be noted, however, that the calculation above assumes a 3Fe cluster. The possibility that some of the Fe detected was free Fe^{2+} , resulting from breakdown of the enzyme to the apoenzyme state, cannot be excluded. In any event, no more than 1 g-atom per 3Fe cluster is required for maximal effect.

Fig. 4 compares the rates of the increases in activity and fluorescence induced by the addition of a low amount of dithionite ($2e^-/3\text{Fe}$) in the

Fig. 4. Comparison of the time courses of the increases in fluorescence and activity induced by dithionite in the presence and absence of Fe^{2+}

Data from the experiments described in Figs. 1–3 were used. The specific activity of the sample after activation with dithionite alone was $6.7 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$; that of the sample with Fe^{2+} plus dithionite was $8.4 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$. The values are expressed as percentage of the maximum change in activity (open symbols) or fluorescence (closed symbols) observed in each time course after the addition of dithionite alone ($2.05e^{-}/3\text{Fe}$; a) or $12.5 \mu\text{M-FeSO}_4$ plus dithionite ($1.96e^{-}/3\text{Fe}$ and $3.5 \text{Fe}^{2+}/\text{Fe}$; b). The rate constants are given in the text.

presence and absence of Fe^{2+} . In the absence of added Fe^{2+} (Fig. 4a), the change in fluorescence developed considerably more quickly than did activity. With an excess of Fe^{2+} present (3.5mol/mol of 3Fe cluster) (Fig. 4b), activity and fluorescence increased simultaneously, following first-order kinetics. The data were fitted to the equation:

$$A = A_0(1 - e^{-kt})$$

where A is the activity or fluorescence at time t , and A_0 is the final value reached. The rate constants for the curves in Fig. 4(b) were $0.71 \pm 0.25 \text{min}^{-1}$ for the activation and $0.694 \pm 0.020 \text{min}^{-1}$ for the increase in fluorescence. For the curves in Fig. 4(a) (no added Fe^{2+}) the rate constants were 0.287 ± 0.044 and $0.535 \pm 0.012 \text{min}^{-1}$ for the activity and fluorescence changes respectively. The fact that in the absence of an external source of Fe^{2+} the development of fluorescence far outstrips the appearance of activity means that the fluorescence change, indicative of a conformational change, precedes the change that confers activity on the enzyme. That the rate of the fluorescence change is but slightly affected by the omission of Fe^{2+} suggests that added Fe^{2+} is not essential for the conformational change that is monitored by fluorescence, although Fe^{2+} may facilitate it to some extent (see also Fig. 5), and is inconsistent with the suggestion that reorganization of the protein is a consequence of insertion of Fe^{2+} (Emptage *et al.*, 1983a).

The difference between the rates of increase in activity and fluorescence depends on the concentration of dithionite used to induce the changes. Fig. 4 shows that as the concentration of dithionite is increased, the initial rate of activation continues to rise after the rate of the fluorescence change reaches a constant value. At high dithionite concentrations ($\geq 8e^{-}/3\text{Fe}$), the rates of the fluorescence and activity changes become equal (as previously observed; Ramsay, 1982), suggesting that the higher concentrations of dithionite promote cluster breakdown, so that Fe is more rapidly made available for insertion into the reduced 3Fe cluster.

Effect of other metal ions

Villafranca & Mildvan (1971) reported several years ago that Fe^{2+} was specific in the activation of aconitase, although Mn^{2+} produced a little activity and competed for the binding site of Fe^{2+} . In contrast, Kennedy *et al.* (1983) found that Mn^{2+} did not affect activation. In our hands, the inclusion of a 8-fold molar excess of either CaSO_4 , MgSO_4 , MnSO_4 , Na_2MoO_4 or NiCl_2 does not affect the activation produced by an 8-fold excess of dithionite. A comparable concentration of ZnSO_4 delayed activation by dithionite, without affecting the final value. Mn^{2+} and MoO_4^{2-} protected the enzyme from the loss of activity which normally occurs overnight in dithionite-activated samples, but the mechanism of this effect is not known. However, the data clearly demon-

Fig. 5. Effect of dithionite concentration on the initial rates of the changes in activity and fluorescence in the presence and absence of Fe^{2+}

Data from the experiments described in Figs. 1–3 were used. Fig. 4 is an example of a set of time courses at one dithionite concentration. The initial rates were calculated from tangents to the time courses between 0 and 0.5 min, and expressed as a percentage of the maximum change in activity (open symbols) or in fluorescence (closed symbols) observed in each series of time courses, either after addition of dithionite alone (circles) or of dithionite + $12.5\text{ }\mu\text{M-Fe}^{2+}$ (squares). After the addition of $10.4e^-$ equivalents/3Fe, the rates of the increases in activity and fluorescence induced by dithionite alone were equal and they remained so at $15.8e^-$ equivalents/3Fe (points not shown).

strate the absolute specificity of the requirement for Fe in the activation of aconitase.

Effect of added sulphide on the activation

Neither the rate nor the extent of activation of mammalian aconitase was influenced by the inclusion of S^{2-} along with Fe^{2+} . This is in agreement with data in the literature (Kennedy *et al.*, 1983), as well as with our analytical data for ten typical preparations of the enzyme from ox heart, as isolated. The range of values found was 2.59 ± 0.30 g-atoms of Fe and 3.60 ± 0.49 mol of 'labile' S per mol of protein (based on a molecular mass of 83 kDa). The resulting Fe/S ratio is 0.73 ± 0.076 . A comparable ratio was found by Gawron *et al.* (1974a) and by Beinert *et al.* (1983). Since the inactive enzyme already contains the four labile S atoms necessary in the active form (Beinert *et al.*, 1983), no further sulphide requirement would be expected.

Is added Fe^{2+} indispensable for activation?

The question whether Fe^{2+} was essential for activation in the presence of a reducing agent has been one of the most difficult ones to answer for this protein. Early studies indicate that maximal activity could be reached by electrochemical reduction without added iron (Ramsay *et al.*, 1981), but it became later apparent that Fe^{2+} could have arisen from the breakdown of the ferrocyanide used as a mediator in these studies. On the other hand, significant, even extensive (up to 75%), activation by dithionite alone was observed under conditions where an external source of iron was rigorously excluded (Beinert *et al.*, 1979; Ramsay, 1982; Singer & Ramsay, 1984). This raised the possibility that the reduced $[\text{3Fe-4S}]$ cluster might also have some activity, an idea reinforced by the m.c.d. data (Johnson *et al.*, 1984). However, the experimental conditions for measuring the activity were such that the extra Fe^{2+} needed to build up the $[\text{4Fe-4S}]$ cluster could have come from what was termed 'cannibalization' of $[\text{3Fe-4S}]$ clusters.

Concurrent studies in the laboratories of Beinert (Kennedy *et al.*, 1983) and in ours on the inclusion of iron chelators during reductive activation without added Fe^{2+} , however, have provided an unambiguous answer. Fig. 6 shows the effect of the inclusion of various concentrations of EDTA on the rate and extent of activation by dithionite. It is seen that, although $2\text{ }\mu\text{M-EDTA}$ had no effect, $20\text{ }\mu\text{M-EDTA}$ completely prevented the activation. Intermediate concentrations decreased the rate of activation to a significant extent. The addition of an excess of Fe^{2+} after the incubation with dithionite and $20\text{ }\mu\text{M-EDTA}$ fully restored maximal activation. It may also be noted that the inclusion of $5\text{ }\mu\text{M-Fe}^{2+}$, along with dithionite in the absence of EDTA, greatly increased the rate of activation, as well as raising the maximal level reached from 65 to 100%.

EDTA had no effect on the activity of activated enzyme in the time observed (Fig. 6), so does not catalyse a 4Fe-to-3Fe cluster conversion (see also Kennedy *et al.*, 1983). To rule out the possibility that the effect of EDTA was due to the formation of a complex with the $[\text{3Fe-4S}]$ form of the enzyme, we treated a sample of inactive aconitase with $200\text{ }\mu\text{M-EDTA}$ for 30 min at 20°C and then separated the low- M_r fraction from the enzyme by centrifugal gel filtration. This preparation was activable by dithionite ($7.5e^-/3\text{Fe}$) to the same extent (50% of maximum) as an untreated control sample.

In contrast with its inhibitory effect on activity, the presence of EDTA during the reduction by dithionite increased the fluorescence change considerably (Fig. 7). Inclusion of Fe^{2+} , on the

Fig. 6. Effect of EDTA on the activation of aconitase by dithionite

The experiment is analogous to that shown in Fig. 1, except that the dithionite concentration was held constant at $17.3 \mu\text{M}$ ($11.9e^-/3\text{Fe}$ in aconitase) and other components were added. The aconitase concentration was $0.30 \text{ mg} \cdot \text{ml}^{-1}$ or $2.9 \mu\text{M}$ (on the basis of Fe determination), and the maximum activity observed in the presence of FeSO_4 was $10.3 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, the same as found in the control activation. The additions, made just before adding dithionite, were: none (dithionite alone; $\circ$), $5 \mu\text{M}$ - FeSO_4 ($\bullet$), $2 \mu\text{M}$ -EDTA ($\square$), $5 \mu\text{M}$ -EDTA ($\blacksquare$), $10 \mu\text{M}$ -EDTA ($\triangle$) or $20 \mu\text{M}$ -EDTA ($\blacktriangle$). FeSO_4 ($25 \mu\text{M}$) added (arrow) after $10 \mu\text{M}$ -EDTA reversed the inhibitory effect on activation. EDTA ($20 \mu\text{M}$) added (downward-pointing arrow) to enzyme activated with dithionite alone had no effect on activity.

other hand, decreased the fluorescence change to 71% of the increment found on activation with dithionite alone (see Fig. 7). In other experiments we have found that the fluorescence increase in the presence of iron varied from 70 to 90% of that for dithionite alone, although activity in the presence of Fe^{2+} was always around 100%. The lower fluorescence change could be partly due to quenching by Fe, but addition of Fe^{2+} to a dithionite-activated sample had no effect on fluorescence. However, in the presence of EDTA, minor effects of added Fe may be observed. Thus addition of $12.5 \mu\text{M}$ -Fe after dithionite reduction in the presence of EDTA caused small increases in fluorescence with $2 \mu\text{M}$ - and $5 \mu\text{M}$ -EDTA, no change with $10 \mu\text{M}$, and a slight decrease with $20 \mu\text{M}$ -EDTA.

Two other iron chelators were also used. Transferrin ($15 \mu\text{M}$ in the presence of bicarbonate), which binds Fe^{3+} , had very little effect on activation or fluorescence, whereas α, α -bipyridyl ($40 \mu\text{M}$), which binds Fe^{2+} , had the same effect on activation and fluorescence as equimolar EDTA. Possible reasons for the divergent effects of Fe^{2+} and of Fe-chelators on activity and fluorescence are considered below in the Discussion section.

Fig. 7. Effect of EDTA on the increase in fluorescence of aconitase induced by addition of dithionite

This Figure shows the relative increase in fluorescence at 333 nm ($\lambda_{\text{ex}} = 295 \text{ nm}$) monitored during the experiment described in Fig. 5. The symbols used indicate the same additions. The arrow indicates addition of $10 \mu\text{M}$ -EDTA at the end of the incubation with dithionite alone. All progress curves where EDTA was present were corrected for this rapid increase in fluorescence not related to the activation.

Discussion

The theoretical number of reducing equivalents for the expected conversions of the oxidized 3Fe cluster is 1, both in the absence and presence of Fe^{2+} .

This stoichiometry was indeed observed when Fe^{2+} was present during activation by dithionite (Fig. 3), but when the titration was carried out in the absence of added iron, the stoichiometry was about $1.8e^-$ per cluster (Fig. 2). The perfect stoichiometry observed in the presence of Fe^{2+} rules out adventitious oxygen or a second reduction step to the $[3\text{Fe-4S}]^-$ form as explanations for the larger amount of dithionite required in its absence. In addition, no evidence has yet been found for the existence of $[3\text{Fe-4S}]^-$ in either e.p.r. or Mössbauer spectroscopy.

The most probable reason for the deviation from unity in the titration with dithionite is that reducing equivalents are required to reduce Fe^{3+} scavenged from other clusters to the Fe^{2+} form required for building up the tetranuclear cluster. It has been proposed that the activity arising on activation with dithionite alone is the result of cannibalization of some of the clusters, which break down to yield the iron required for insertion into the trinuclear cluster (Kennedy *et al.*, 1983; Emptage *et al.*, 1983a). Since the maximal activity we obtain with dithionite is about 75% of that observed when both dithionite and Fe^{2+} are present, one may assume that 25% of the $[3\text{Fe-4S}]^0$ clusters resulting from reduction of the $[3\text{Fe-4S}]^+$ clusters were destroyed to yield the extra iron required to build up 75% of the clusters to the $[4\text{Fe-4S}]^{2+}$ form. Each of the 3Fe clusters destroyed yields one Fe^{2+} and two Fe^{3+} , so that 25% of the clusters initially present would require a 50% excess of dithionite for full reduction of the liberated Fe. Hence, maximal activation in the absence of added Fe^{2+} would require 1.5 reducing equivalents per cluster initially present. The experimental finding of ~ 1.8 equivalents per cluster might indicate contributions from the other sources, such as Fe that is liberated from 4Fe clusters on oxidation but remains with the protein after gel filtration (Kennedy *et al.*, 1983).

Although this explanation seems to be the most plausible among those discussed, it leads to yet other unanswered questions. The concept of cannibalization supposes that the clusters break down all the way to the apoenzyme. If so, the process must be fully reversible, since on addition

of Fe^{2+} to dithionite-activated aconitase, the activity increases to the same level as would be observed if dithionite plus Fe^{2+} were simultaneously present during activation. The question arises as to what happens to the sulphur on breakdown of the cluster, so that none needs to be added subsequently to reconstruct the $[4\text{Fe-4S}]^{2+}$ cluster. Kennedy *et al.* (1983) have recently shown that S^{2-} remains bound to the protein during cluster breakdown. In contrast, the requirement for added Fe^{2+} to yield the fully active form is clear, and the amount of Fe required for this (0.6g-atoms per 3Fe) is in line with some Fe having been retained on the protein during oxidation as discussed above.

Whereas it is established that full activation involves the conversion of a $[3\text{Fe-4S}]^+$ cluster in the oxidized enzyme (Kent *et al.*, 1982; Beinert *et al.*, 1983) into the full active $[4\text{Fe-4S}]^{2+}$ form (Kent *et al.*, 1982; Emptage *et al.*, 1983a; Johnson *et al.*, 1983, 1984), it may be premature to conclude that it is the only form endowed with any activity. Reduction of the active enzyme to the $[4\text{Fe-4S}]^+$ form as detected by e.p.r. spectroscopy is associated with a significant, but not complete, decrease in activity (Emptage *et al.*, 1983a). In addition, there are indications from m.c.d. experiments that the $[3\text{Fe-4S}]^0$ form of the cluster may endow partial activity on the enzyme.

The strongest evidence in favour of the proposal that Fe liberated from other clusters is used to build up the $[4\text{Fe-4S}]^{2+}$ cluster during activation by dithionite comes from the fact that EDTA, which does not inhibit the active enzyme, prevents activation by dithionite (Fig. 6). The obvious interpretation is that EDTA 'mops up' any Fe^{2+} released from the destruction of trinuclear clusters.

The effect of EDTA on the fluorescence change (Fig. 7) associated with the conformational alteration during activation can also be interpreted in terms of the formation of a $[4\text{Fe-4S}]$ cluster. The fully active form, which arises in the presence of dithionite plus Fe^{2+} , has a lower fluorescence than the partly active form obtained with dithionite alone. The enhancement of EDTA is consistent with the prevention of the formation of tetranuclear clusters, leaving the enzyme in the more fluorescent reduced trinuclear state ($[3\text{Fe-4S}]^0$).

The difference in the rate of reduction of the Fe-S cluster, which is very rapid [less than the mixing time (Ramsay, 1983) and less than 2ms when $10e^-$ equivalents of dithionite were mixed with aconitase in the stopped-flow apparatus (R. R. Ramsay, unpublished work)] and the slow increase in fluorescence demonstrate that the fluorescence change is not merely due to the changes in absorbance of the iron-sulphur cluster. The protein undergoes an alteration in the region of the Fe-S cluster whether or not its trinuclear cluster is

built up to a tetranuclear one. However, activation does not take place in the presence of EDTA, so that activity must be dependent on the insertion of the fourth Fe atom, as demonstrated by Kennedy *et al.* (1983) and Emptage *et al.* (1983a,b). The fluorescence change thus reflects reorganization in the cluster site to facilitate the insertion of Fe and is not a consequence of Fe insertion, as previously suggested (Emptage *et al.*, 1983a).

That the conformational change precedes Fe insertion is deduced from the difference between the rates of the increase in fluorescence and activity at low dithionite concentrations (Figs. 4 and 5). We previously reported identical rates for the increases in fluorescence and activity (Ramsay, 1982) at a dithionite level of $14e^-$ per mol of aconitase. This remains valid, the difference in rates being observed only up to $8e^-$ per 3Fe cluster. It is probable that the higher concentrations of dithionite used in the early experiments enhanced the breakdown of clusters, making Fe^{2+} available rapidly enough for the reconstruction of the 4Fe clusters as fast as the protein assumed the correct conformation.

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