Evidence that the activation of aconitase involves a conformational change

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Reduction of the iron–sulphur cluster of aconitase initiates a slow increase in catalytic activity. It has been proposed that activation involves a conformational change in the protein. Direct evidence for this is presented here in the demonstration that, after reduction of the cluster, the progressive increase in activity is paralleled by an increase in the fluorescence of the protein.

Aconitase (aconitate hydratase, EC 4.2.1.3) is an unusual enzyme in that it contains an iron–sulphur cluster, although it does not catalyse a redox reaction (Ruzicka & Beinert, 1978a). The iron–sulphur moiety is a 3Fe–3S cluster (Emptage *et al.*, 1980; Kent *et al.*, 1981), but it may collapse to a binuclear cluster or be built up to a tetranuclear one under appropriate conditions (Kurtz *et al.*, 1979; Ramsay *et al.*, 1981). The cluster is reduced and e.p.r.-silent in the active enzyme (Ruzicka & Beinert, 1978a), but in the inactive oxidized state an e.p.r. signal at $g = 2.01$ (characteristic of high-potential Fe–S proteins) is present. Conversion between the two forms requires chemical or electrochemical oxidation or reduction of the iron–sulphur cluster (Gawron *et al.*, 1974; Ruzicka & Beinert, 1978b; Beinert *et al.*, 1979; Ramsay *et al.*, 1981). The redox change alters the absorbance and e.p.r. signal of the Fe–S cluster, changes which occur rapidly, whereas the change in catalytic activity shows a pronounced lag (Beinert *et al.*, 1979; Ramsay *et al.*, 1981). These observations, together with binding studies, led to the suggestion that the loss and appearance of activity were consequences of conformational changes in the protein (Ramsay *et al.*, 1981).

To test the proposal, the change in the fluorescence of the tryptophan residues of aconitase has been correlated with the appearance of catalytic activity. This method has often been used to investigate structural alterations in proteins (Brand & Witholt, 1967; Lakowicz, 1980). It was found that the fluorescence of aconitase increased in parallel with the catalytic activity, both reaching a maximum at the same time after reduction of the iron–sulphur cluster.

Experimental

Aconitase was prepared from ox heart mitochondria

as previously reported (Ramsay *et al.*, 1981). Before use, samples were passed through aerobic columns of Sephadex G-50 (fine beads) in 25 mM-potassium phosphate, pH 7.4, by the method of Penevsky (1977), to remove dithioerythritol and to oxidize the aconitase. Analytical and activity assays were as described previously (Ramsay *et al.*, 1981).

Absorbance spectra were recorded on a Cary 219 spectrophotometer. Fluorescence-emission spectra and intensities were measured by using a Perkin–Elmer fluorescence spectrophotometer with corrected spectra accessory (model MPF-3). The temperature of the cell holder was kept at 15°C by circulating water. The excitation wavelength was 295 nm and corrected emission spectra were recorded between 300 and 400 nm. Aconitase (to give 0.5 mg · ml⁻¹, and an A_{295} of 0.28) was added to anaerobic buffer (4 ml; 25 mM-potassium phosphate, pH 7.4) in a closed fluorescence cell (1 cm) with a side arm connected to a syringe containing dithionite (4–10 mM; the exact concentration determined by titration against 40 μ M-riboflavin). The fluorescence intensity at the emission maximum (333 nm) was recorded for 15 s each minute. The sample was not illuminated between readings, although continuous illumination made no apparent difference. Changes in fluorescence intensity were calculated relative to the intensity of oxidized aconitase. The cell was fixed into position and connected to a flow of argon so that the stopper could be removed to permit mixing by a stream of argon through polyethylene tubing (0.38 mm internal diameter) and sampling by micropipette without loss of anaerobiosis. Excess sample was withdrawn to protect the enzyme (10 μ l) for assay from oxygen during the transfer. In some experiments 5 mM-glucose, glucose oxidase (1 unit · ml⁻¹; reagent grade; Miles Laboratories) and catalase (24 units · ml⁻¹; Sigma Chemical Co.) were included, but their inclusion made no significant difference.

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Results and discussion

The fluorescence emission spectrum of aconitase (Fig. 1) has a maximum at 333 nm. The quantum yield, by comparison with tryptophan (0.14), was 0.04 at 15°C. (All experiments were conducted at 15°C, because at that temperature the activity of aconitase increased slowly enough to be monitored conveniently.) Addition of portions of dithionite to reduce the iron-sulphur centre of aconitase increased the fluorescence intensity (Fig. 1), but the emission maximum did not change. Therefore the fluorescence intensity was measured as the peak height at this wavelength in all subsequent experiments. The maximum enhancement of about 15% was achieved after addition of a 3-fold excess of dithionite (a 6-fold excess of reducing equivalents) and was not further increased by up to a 10-fold excess of dithionite (76 μM for the experiment shown in Fig. 1), after correction for the

Fig. 1. Corrected emission spectra of the intrinsic fluorescence of oxidized and reduced aconitase. Spectra of oxidized (—) and reduced (----) aconitase were recorded anaerobically at 15°C as described in the Experimental section. The spectrum of reduced aconitase was taken 10 min after addition of 66.2 nmol of dithionite to 22.8 nmol of aconitase in 3 ml of buffer (7.6 μM -aconitase is 0.63 $\text{mg} \cdot \text{ml}^{-1}$).

absorbance of the exciting light by dithionite. With free tryptophan (10 μM) or bovine serum albumin (0.5 $\text{mg} \cdot \text{ml}^{-1}$) there was only slight quenching (<10%) of the emission spectra up to 76 μM -dithionite. In neither case was there any enhancement of fluorescence at low dithionite concentrations (e.g. 22 μM). Therefore the enhancement of the fluorescence of aconitase appears to be a specific property associated with the activation that occurs on reduction of the enzyme.

Fig. 2 shows that the amount of dithionite (3.6 mol/mol of aconitase, i.e. 7 electron equivalents) required to achieve the maximum increase in fluorescence is the same as that required to reach minimum absorbance at 480 nm (which reflects complete reduction of the iron-sulphur centre) and to elicit maximum activity. It is clear from the correlation of the titration of these three variables that they are directly related. The requirement for an excess of dithionite to reduce the iron-sulphur cluster of aconitase (in theory a 1-electron reduction) was noted previously (Beinert *et al.*, 1979), but the reason is not clear. It is not due to residual oxygen in the system, because parallel titration of riboflavin showed immediate reduction, and the inclusion of the oxygen-scavenging system (glucose oxidase) in the aconitase experiment did not decrease the amount of dithionite required.

Although the titration curves with dithionite are identical for absorbance, fluorescence and activity changes (Fig. 2), these parameters change at different rates after the addition of dithionite. The absorbance change at 480 nm was complete within the mixing time (not shown; see Ramsay *et al.*,

Fig. 2. Titration of aconitase with dithionite. Aconitase (0.7 $\text{mg} \cdot \text{ml}^{-1}$, 8.4 μM) was titrated with 4.44 mM-dithionite as described in the Experimental section. The difference from the starting sample was measured 10 min after each addition of dithionite, and expressed as percentage of the final maximum difference. Activity (O), fluorescence at 333 nm (Δ) and A_{480} ($\square$) were monitored.

1981), but the activity and fluorescence changes developed slowly, as shown in Fig. 3, reaching completion after 10 min at 15°C. The time course for the appearance of activity coincides with the increase in fluorescence, suggesting that both are consequences of the same event, presumably a conformational change in the protein.

It should be noted that these changes can be initiated by reductant alone, as has been reported recently for activation of the enzyme (Ruzicka & Beinert, 1978b; Ramsay *et al.*, 1981). In the past, activation of aconitase was reported to require Fe and thiol (Villafranca & Mildvan, 1971; Gawron *et al.*, 1974), but the aconitase used was deficient in iron. The preparations of aconitase used in the present study contained the full complement of 2.7–3.0 g-atoms of Fe per mol of aconitase. The experiments shown in Figs. 2 and 3 support the premise (Ramsay *et al.*, 1981) that, although the reduction of the iron–sulphur centre is all that is required to elicit maximal activity, the appearance of activity is delayed because a slow conformational change must occur in the protein before activity is expressed.

It must be emphasized that the conformational change discussed here is that which occurs during activation of the enzyme. Another, totally distinct, conformational change takes place in the presence of

substrate, namely that which occurs during the catalytic cycle of all enzymes to greater or lesser extent. In aconitase the catalytic conformational change can also be observed by fluorescence. When citrate is added to the active enzyme, a decrease in fluorescence occurs that does not happen when citrate is added to tryptophan or bovine serum albumin (results not shown). The quenching (~7%) reaches a maximum at 1 mM-substrate (about $5 \times K_m$). This change is clearly different from the one taking place during activation; the former quenches fluorescence (by 7%), the latter enhances fluorescence (by 15%).

These experiments suggest that fluorescence could prove to be a useful technique for studying the changes that take place during the activation of aconitase. It should be possible to compare the progress and extent of the conformational changes in the protein during activation by chemical or coulometric means and, hence, explore why dithionite gives only about 80% activation compared with other methods, and why the spectral changes are not completely reversible (Beinert *et al.*, 1979).

The present experiments provide the first direct evidence that a conformational change is required to convert inactive aconitase into the active form. They confirm that reduction of the iron–sulphur centre is all that is necessary for the activation (Ramsay *et al.*, 1981), but the conformational change that occurs as a result of the reduction is the rate-limiting step for the appearance of activity.

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Fig. 3. Time course of the increase in fluorescence and activity of aconitase after reduction by dithionite. Dithionite (169 nmol) was added to 24 nmol of aconitase ($0.5 \text{ mg} \cdot \text{ml}^{-1}$), mixed, and fluorescence intensity at 333 nm noted every minute. Samples ($10 \mu\text{l}$) were assayed as described in the Experimental section. The changes are expressed as percentage of the maximum difference between oxidized and reduced enzyme, which was $7.2 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ for activity (○) and 14% of the peak height for fluorescence (△).

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