Rapid inactivation of plant aconitase by hydrogen peroxide

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Preincubation of potato (*Solanum tuberosum*) tuber mitochondria with 300 μM-H_2O_2 for 10 min nearly stopped the State 3 rate of citrate oxidation. Addition of isocitrate resulted in resumption of O_2 uptake. The State 3 rates of succinate, external NADH and 2-oxoglutarate oxidation were unaffected by H_2O_2 over the dose range 50–500 μM. Preincubation of mitochondria with 300 μM-H_2O_2 for 5 min unmasked in the matrix space a paramagnetic signal with a peak at a g value of approx. 2.03. Aconitase was purified over 135-fold to a specific activity of 32 μmol/min per mg (with isocitrate as substrate) from the matrix of potato tuber mitochondria. The native enzyme was composed of a single polypeptide chain (molecular mass 90 kDa). Incubation of purified aconitase with small amounts of H_2O_2 caused the build up of a paramagnetic 3Fe cluster with a low-field maximum of $g = 2.03$ leading to a progressive inhibition of aconitase activity. The results show that aconitase present in the matrix space was the major intramitochondrial target for inactivation by H_2O_2.

INTRODUCTION

Aconitase [aconitate hydratase; citrate (isocitrate) hydrolyase, EC 4.2.1.3] catalyses the reversible hydration of *cis*-aconitate to either citrate or isocitrate. Active aconitase from muscle has been characterized as an iron–sulphur protein, containing a single 4Fe–4S cubic cluster per 90 kDa enzyme (Kent *et al.*, 1982). Electron-nuclear-double-resonance spectroscopy and Mössbauer-effect studies using ^{17}O-labelled substrates and inhibitors support a mechanism in which the Fe–S cluster simultaneously binds a hydroxy group of the substrate (isocitrate, citrate or *cis*-aconitate), one carboxy group of the substrate and a water molecule (Kennedy *et al.*, 1987; Emptage, 1988). The probable physiological state of the iron–sulphur cluster in mammalian aconitase is $[4Fe\text{–}4S]^{2+}$, which is diamagnetic. However, during oxidation or purification of aconitase one Fe is lost, leaving a paramagnetic $[3Fe\text{–}4S]^{+}$ cluster that is completely inactive [for a review, see Emptage (1988)]. This mobile iron is also the iron site which forms a complex with substrates.

In plants, aconitase has been characterized in several tissues [for a review, see Pickworth Glusker (1971)]. Fairly recently, Brouquisse *et al.* (1986, 1987) demonstrated that at least two aconitase isoenzymes are present in intact plant cells: one is readily released after stripping the cell membrane and is present in the cytosol; the other is confined within mitochondria. Although the isolation of aconitase was not carried out, it appeared that the aconitase in plant mitochondria differs from that of mammalian mitochondria in several respects. For example, the e.p.r. spectrum observed in the oxidized mitochondrial matrix from potato (*Solanum tuberosum*) tubers resembles that of the oxidized form of ox liver cytoplasmic aconitase, but differs clearly from the spectra of ox liver and pig heart mitochondrial aconitase (Beinert & Thompson, 1983).

With the aim of further clarifying the structure and function of plant aconitase, the present paper details the rapid isolation of aconitase from potato tuber mitochondria. In addition we report the strong effects of a catalytic amount of H_2O_2 on plant mitochondrial aconitase activity, including the building up of a paramagnetic 3Fe cluster.

MATERIAL AND METHODS

Isolation of mitochondria

Mitochondria were isolated and purified from potato tubers as described by Douce *et al.* (1987) by using self-generating Percoll gradients. The mitochondria were found in a broad brown–red band near the bottom of the tube, whereas the 'microsomal' fraction, containing the yellow envelope surrounding the amyloplasts, remained near the top of the tube. The mitochondria were subsequently concentrated by differential centrifugation. In order to remove all the contaminating peroxisomes and, therefore, catalase activity, the purification procedure was repeated once (i.e. a second time through Percoll). Under these conditions we have verified that catalase activity with substrate concentrations up to 200 μM was almost negligible. The purified mitochondria were suspended in a medium (suspending medium) containing 0.3 M-mannitol, 10 mM-phosphate buffer, pH 7.2, and 1 mM-EDTA at approx. 50–100 mg of protein/ml. O_2 uptake was measured at 25 °C with a Clark-type oxygen electrode purchased from Hansatech (King's Lynn, Norfolk, U.K.). The reaction medium (electrode medium) contained 0.3 M-mannitol, 5 mM-$MgCl_2$, 10 mM-KCl, 10 mM-phosphate buffer, pH 7.2, 0.1 % (w/v) defatted BSA and known amounts of mitochondrial protein in a total volume of 1 ml. With 5 mM-citrate as substrate, the respiratory-control index was routinely better than 2 and the State 3 rate of O_2 uptake was higher than 70 nmol/min per mg of protein. The mitochondria were more than 95% intact as judged by their impermeability to cytochrome *c* (Douce *et al.*, 1987).

Purification of aconitase

Potato tuber mitochondria (about 1.2 g of protein) were diluted in 125 ml of lysis buffer containing 10 mM-Tris, pH 7.2, 1 mM-citrate and 1 mM-EDTA. Citrate is required to protect aconitase activity. The addition of 1 mM-phenylmethanesulphonyl fluoride, 1 mM-benzamidine and 5 mM-hexanoic acid to the lysis buffer provides further protection of aconitase against endogenous proteinases. Total release of the matrix protein was achieved by three cycles of freezing and thawing. The mitochondrial sus-

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pension was frozen by placing it at 77 K for 5 min. The frozen mitochondrial suspension was then maintained at 30 °C until thawed. This procedure breaks about 98 % of the mitochondria. The suspension of broken mitochondria was centrifuged at 100000 *g* for 2 h (35000 rev./min in a Sorvall A.641 rotor) to remove all the mitochondrial membranes. The supernatant thus obtained (120 ml; 4 mg of protein/ml) was filtered through a 0.22 μm-pore-size filter (Sterivex-GS, Millipore).

Crystalline $(NH_4)_2SO_4$ was added to the matrix extract (480 mg of protein) with stirring until 50 % saturation was achieved, and the extract was left for 1 h at 4 °C. The precipitate was removed by centrifugation at 20000 *g* for 30 min (Sorvall SS-34 rotor). Crystalline $(NH_4)_2SO_4$ was added to the supernatant with stirring until 70 % saturation was achieved and the extract was left for 1 h at 4 °C. The precipitate was collected by centrifugation (20000 *g*, 30 min) and resuspended in a minimal volume of medium A [10 mM-Tris/HCl (pH 7.5)/1 mM-citrate].

The protein suspension was applied to a Trisacryl M DEAE column (Industrie Biologique Française; 1 cm × 20 cm) connected to a Pharmacia f.p.l.c. system and previously equilibrated with medium A. Aconitase was eluted at 4 °C with a continuously increasing Tris/citrate gradient (pH 7.2; 0–250 mM) (Flow rate 0.5 ml/min; fraction size 1.5 ml). Fractions containing activity were pooled and concentrated to a final volume of 1 ml by ultrafiltration on a Diaflo membrane XM-50 using a stirred cell on a magnetic-stirring table (Amicon).

The concentrated sample was applied to a Mono Q HR 10/10 column (Pharmacia; 1 cm × 10 cm) previously equilibrated with medium A. The column, connected to a Pharmacia f.p.l.c. system, was eluted with a linear KCl gradient in medium A (0–200 mM) (flow rate 1.5 ml/min; fraction size 1 ml). Aconitase emerged as a sharp peak at 80 mM-KCl. Peak fractions were combined and concentrated to a final volume of 1 ml by ultrafiltration.

The concentrated enzyme was applied to a Procion Red column (Amicon; 0.5 cm × 10 cm) previously equilibrated with medium A. The column connected to a Pharmacia f.p.l.c. system was eluted with a linear KCl gradient in medium A (0–1 M). Fraction containing activity were pooled and concentrated to a final volume of 1 ml by ultrafiltration.

The concentrated enzyme solution from the previous step and containing 1.1 M-$(NH_4)_2SO_4$ was applied to a Phenyl-Superose HR 5/5 column (Pharmacia; 0.5 cm × 5 cm) previously equilibrated with medium A containing 1.1 M-$(NH_4)_2SO_4$. The column, connected to a Pharmacia f.p.l.c. system, was eluted with a decreasing $(NH_4)_2SO_4$ gradient (1.1–0 M) (flow rate 0.5 ml/min; fraction size 1 ml). Aconitase emerged as a peak at 0.8 M-$(NH_4)_2SO_4$. Fractions containing activity were pooled and concentrated to a final volume of 1 ml by ultrafiltration.

Purified aconitase could be stored at −80 °C in medium A containing 200 mM-$(NH_4)_2SO_4$ under N_2 without deterioration for many months. During the course of aconitase purification we have observed that, in the presence of $(NH_4)_2SO_4$, aconitase activity was greatly stimulated.

E.p.r. measurements

These were recorded on a Varian E 109 spectrometer coupled to a Hewlett–Packard 9826 computer. The samples were cooled with a liquid-helium transfer system (Oxford Instruments; ESR 900) for variable temperatures down to 4.2 K. Temperature was monitored with a gold–iron/chromel thermocouple about 2 cm below the bottom of the e.p.r. sample in the flowing helium-gas stream. The magnetic field was calibrated using a Varian gaussmeter. Samples of mitochondria or aconitase fraction concentrated by ultrafiltration through a low-absorption hydrophilic YM membrane (30000-M_r cut-off centricon; Amicon) were placed in e.p.r. quartz tubes, frozen rapidly in liquid N_2, and stored at 77 K until assayed.

Assay of aconitase

All assays were optimized with respect to the concentration of each component and to the pH of the reaction mixture. The reactions were monitored with a spectrophotometer (Kontron Uvikon 810), and the coupling enzyme system was checked not to be rate-limiting. Aconitase activity was assayed at 25 °C in a final volume of 0.5 ml, and the buffer used was either 50 mM-Tricine/NaOH (for pH values above 7.4) or 50 mM-Mops/NaOH (for pH values below 7.4). Aconitase activity was assayed either by the coupled assay of Rose & O'Connel (1967) in which $NADP^+$ reduction is measured, or by monitoring the formation of *cis*-aconitate at 240 nm as a function of time (Fansler & Lowenstein, 1969).

Electrophoresis

SDS/PAGE was performed at 4 °C in SDS/polyacrylamide slab gels containing a linear 7.5–15 % (w/v) acrylamide gradient. The experimental conditions for gel preparation, sample solubilization, electrophoresis and gel staining were detailed by Chua (1980). Native gels, consisting of 7.5 % acrylamide and 0.2 % bisacrylamide in 40 mM-Tris/HCl, pH 8, were prepared as described by Laemmli (1970), except that detergent was omitted from the different buffers. Electrophoresis was performed at 15 mA/gel at 4 °C for 6 h.

RESULTS

Effect of H_2O_2 on citrate oxidation by potato tuber mitochondria

Fig. 1(*a*) indicates that, in the presence of 200 μM-NAD^+, potato tuber mitochondria oxidize citrate. Stimulation of respiration in isolated plant mitochondria by exogenous NAD^+ is well known (Douce & Neuburger, 1989). NAD^+ is accumulated by potato tuber mitochondria via a specific carrier, and it is now generally agreed that stimulation of O_2 uptake by added NAD^+ is due to its stimulation of matrix-localized enzymes. Fig. 1 also indicates that, during the course of citrate oxidation, these mitochondria display depressed initial State 3 rates of respiration, which rise to a maximum with several consecutive State 3/State 4 cycles. By preincubating the mitochondria with a small amount of malate (result not shown; see Journet & Douce, 1983), the maximal rate of O_2 consumption is attained more rapidly because isolated potato tuber mitochondria appear to be depleted of exchangeable anions (citrate is transported inward on a citrate carrier and phosphate outward on a dicarboxylate carrier with malate as the counter-ion on both translocators in a cascade-like manner).

Since inhibition of citrate oxidation by H_2O_2 is a function of both the concentration of the peroxide and the time of its preincubation with the mitochondria, IC_{50} (concn. causing 50 % inhibition) values cannot be calculated in a simple manner. Hence the following procedure was adopted. The mitochondria, devoid of catalase activity and suspended in electrode medium, were preincubated in the polarographic chamber at 25 °C with several concentrations of H_2O_2 for various times from 0 (control experiment) to 15 min. At the end of the incubation, NAD^+, ADP and citrate were added, and the rate of O_2 consumption was determined at 25 °C as described above. An oxygen-electrode trace showing the effect of H_2O_2 addition on coupled potato tuber mitochondria oxidizing citrate is presented in Fig. 1. Preincubation of mitochondria with 300 μM-H_2O_2 for 10 min nearly stopped State 3 citrate oxidation (Fig. 1*b*). However, addition of 10 mM-isocitrate to the oxygen-electrode medium

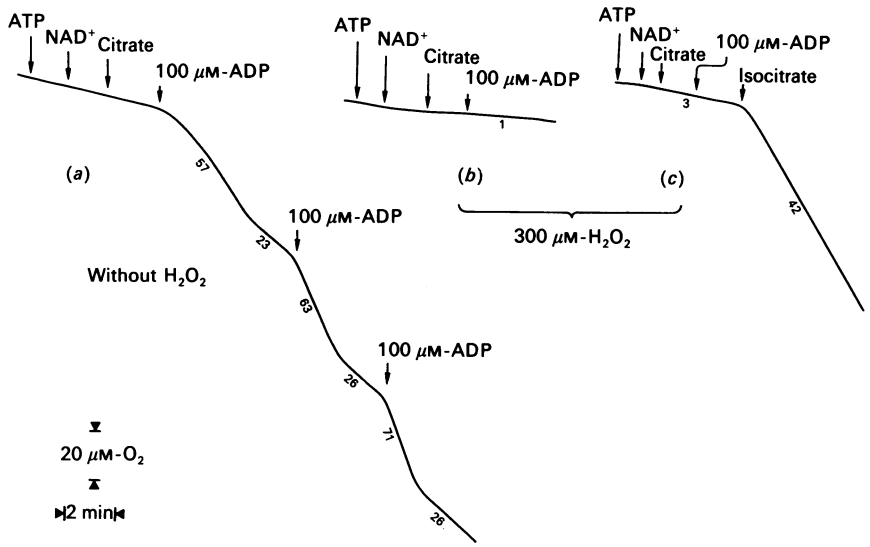

Fig. 1. Influence of H_2O_2 on citrate oxidation by purified potato tuber mitochondria

Mitochondria were suspended to an appropriate protein concentration (0.37 mg/ml) in electrode medium and were preincubated with (*b*, *c*) or without (*a*) 300 μM-H_2O_2 for 10 min. Citrate (10 mM) and appropriate cofactors (300 μM-ATP, 200 μM-NAD^+ and 100 μM-ADP) were added. Where indicated, 10 mM-isocitrate was added (*c*). O_2-uptake measurements and the reaction mixture were as described in the Materials and methods section. Values on the traces refer to nmol of O_2 consumed/min per mg of protein. Note that isocitrate oxidation is not affected by H_2O_2. Since most of the plant mitochondria isolated so far are depleted of their endogenous NAD^+, it is necessary to add this cofactor in the medium in order to trigger the full rate of citrate oxidation.

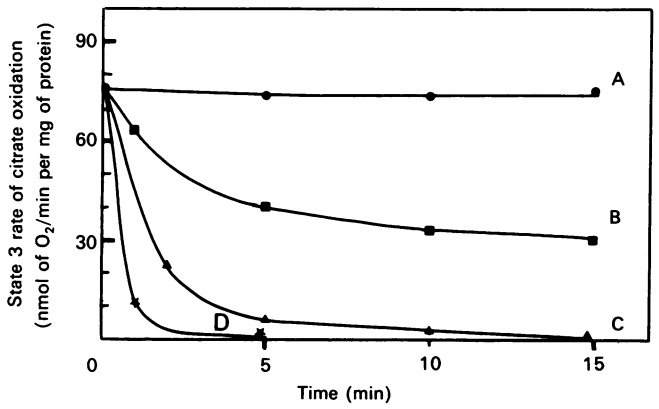

Fig. 2. Time course of the development of inhibition of State 3 rate of citrate oxidation with H_2O_2

Mitochondria were suspended to an appropriate protein concentration (0.44 mg/ml) in electrode medium and were preincubated with H_2O_2 at various concentrations. At the time indicated, 1 ml aliquots were transferred to an oxygen-electrode vessel and O_2 uptake was measured in the presence of 10 mM-citrate and appropriate cofactors (300 μM-ATP, 200 μM-NAD^+ and 1 mM-ADP). A, control experiment; B, 100 μM-H_2O_2; C, 300 μM-H_2O_2; D, 500 μM-H_2O_2.

resulted in resumption of O_2 uptake (Fig. 1*c*). At external concentrations above 500 μM the inhibitory effect of H_2O_2 was virtually instantaneous (Fig. 2). On the other hand, at external concentrations lower than 500 μM the development of inhibition of citrate oxidation was progressive, and the time of preincubation with H_2O_2 required to reach 50% inhibition of State 3 respiration increased as the concentration of H_2O_2 decreased. In addition, removal of H_2O_2 by washing with 0.3 M-mannitol medium or by adding a large excess of catalase, did not restore citrate oxidation (result not shown). It is emphasized, however, that the effectiveness of H_2O_2 in blocking citrate oxidation in intact mitochondria was strongly reduced by citrate. For example, inhibition of citrate oxidation was not observed, even at 500 μM external concentration of H_2O_2, if 10 mM citrate was added before H_2O_2. In contrast, isocitrate was without effect on the ability of H_2O_2 to block citrate oxidation (result not shown). Interestingly, we have observed that the State 3 rate of succinate, external NADH and 2-oxoglutarate oxidation were unaffected by H_2O_2 over the dose range 50–500 μM. Taken together, these results suggest that low concentrations of H_2O_2, in the absence of citrate, irreversibly damage the components of citrate oxidation (tricarboxylate carrier? aconitase?). Since H_2O_2 was without effect on isocitrate oxidation, we can conclude that the tricarboxylate carrier, which transports isocitrate as well as citrate (Day & Wiskich, 1984), was not involved in the inhibition of citrate oxidation by H_2O_2. Experiments were therefore undertaken to see whether the inhibition of citrate oxidation was attributable to a direct effect of H_2O_2 on aconitase present in the matrix space.

Effect of H_2O_2 on the e.p.r. characteristics of aconitase in intact plant mitochondria

Fig. 3 shows e.p.r. spectra recorded at 10 K of intact oxidized mitochondria. An intense and nearly isotropic e.p.r. signal was seen at $g = 2.014$ with a low-field maximum of $g = 2.02$ (Fig. 3*a*). This signal was also found in submitochondrial particles (Brouquisse *et al.*, 1987). By contrast, this signal was not detected in the high-speed supernatant of sonicated mitochondria (Brouquisse *et al.*, 1987). This type of signal is of the type expected for a 3Fe cluster in the oxidized state ($S = 1/2$) and assigned to centre S_3 in succinate dehydrogenase (Beinert *et al.*, 1975). Preincubation of mitochondria with 300 μM-H_2O_2 for 1 min before freezing in liquid N_2 unmasked a second signal with a peak at a g value of approx. 2.03, causing an apparent broadening of the $g = 2.02$ (centre S_3) peak (Fig. 3*b*). The maximum enhancement was achieved after addition of 100 μM-H_2O_2 and was not increased further by up to 500 μM-H_2O_2. This second iron–sulphur centre distinct from centre S_3 was readily removed from the mitochondrial membrane during the disruption of mitochondria and subsequent isolation of the matrix fraction

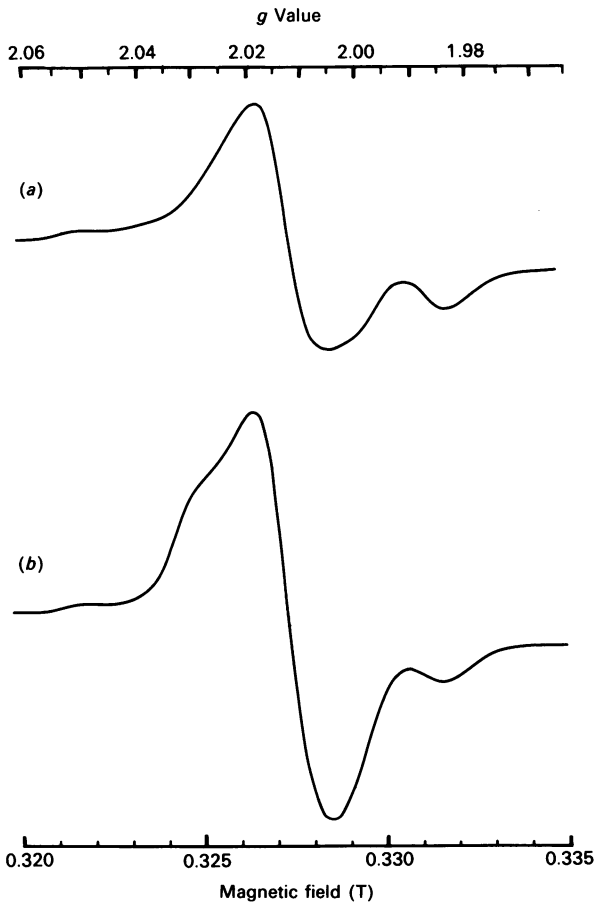

Fig. 3. First-derivative e.p.r. spectra of intact mitochondria isolated from potato tubers

Mitochondria were resuspended to an appropriate protein concentration (55 mg/ml) in an electrode medium devoid of $MgCl_2$. (*a*) Control experiment. O_2 was blown over the suspension for 10 s before it was transferred to a quartz e.p.r. tube and frozen at 77 K. (*b*) Mitochondria were preincubated with 300 μM-H_2O_2 for 1 min before freezing. Conditions of measurement were: modulation amplitude, 0.8 mT; modulation frequency, 100 kHz; microwave frequency, 9252 MHz; microwave power, 0.5 mW; temperature, 10 K.

and submitochondrial particles (results not shown; see Brouquisse *et al.*, 1986). The e.p.r. spectrum of the soluble fraction obtained from H_2O_2-treated mitochondria revealed the presence of a nearly isotropic signal with a low-field maximum at $g = 2.03$, which identifies the 3Fe cluster. This spectrum has a shoulder in the centre of the resonance absorbance (Brouquisse *et al.*, 1986). A similar signal in the high-speed supernatant of sonicated mitochondria isolated from mammalian tissues was observed by Ohnishi *et al.* (1976); this signal was later attributed to aconitase by Ruzicka & Beinert (1978). When the isotropic signal with a low-field maximum at $g = 2.03$ was fully unmasked, aconitase activity measured in the matrix fraction was not detectable. These results strongly suggest that inhibition of citrate-dependent O_2 consumption in plant mitochondria by H_2O_2 is attributable to a mild oxidative inactivation of matrix aconitase.

Action of H_2O_2 on aconitase isolated from potato tuber mitochondria

The overall scheme and results of the purification procedure for mitochondrial aconitase are shown in Table 1. The enzyme

Table 1. Purification of aconitase from potato tuber mitochondria

Aconitase activity was assayed by monitoring the formation of *cis*-aconitate at 240 nm (Fansler & Lowenstein, 1969). These data are from a representative experiment.

Purification stage	Total protein (mg)	Total activity (μmol/min)	Specific activity (μmol/min per mg)	Yield (%)
Broken mitochondria	1200	290	0.25	100
Matrix fraction	480	264	0.55	91
Trisacryl M DEAE pool	62	133	2.15	46
Mono Q HR 10/10 pool	5	31.7	6.35	11
Procion Red pool	1	9.73	9.73	3
Phenyl-Superose HR 5/5 pool	0.3	9.72	32.1	3

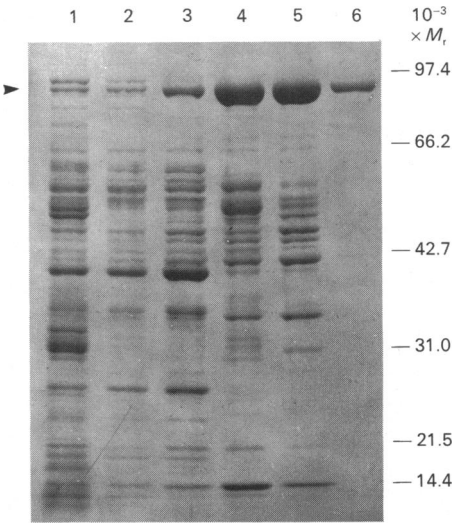

Fig. 4. SDS/PAGE of samples from successive purification steps of mitochondrial aconitase

Proteins were separated on an SDS/7.5–15% (w/v)-gradient-polyacrylamide slab gel stained with Coomassie Brilliant Blue R-250. Lane 1, broken mitochondria, 10 μg; 2, matrix fraction, 8 μg; 3, Trisacryl M DEAE pool, 12 μg; 4, Mono Q HR 10/10 pool, 15 μg; 5, Procion Red pool, 15 μg; 6, Phenyl-Superose pool, 3 μg. The arrow indicates the position of aconitase.

was purified 130-fold and the yield was greater than 3%. After the subsequent ion-exchange-chromatography and affinity-chromatography steps, aconitase was almost pure, with a molecular mass of 90 kDa as determined by SDS/PAGE (Fig. 4). The native molecular mass of aconitase was estimated to be 90 kDa using PAGE under non-denaturing conditions. These results strongly suggest that plant mitochondrial aconitase, like its mammalian counterpart (Ryden *et al.*, 1984), consists of a single polypeptide chain. Plant mitochondrial aconitase is fully active and does not require activation by anaerobic incubation for 30 min at room temperature with an excess of Fe^{2+} and a thiol such as cysteine or dithioerythritol [for a review, see Emptage (1988)]. Furthermore, whenever plant mitochondrial aconitase is inactivated during the course of its purification (the activity of the form isolated depends on the speed of isolation, the degree of anaerobiosis maintained and the temperature of conservation), attempts to re-activate the enzyme using Fe^{2+} and cysteine were

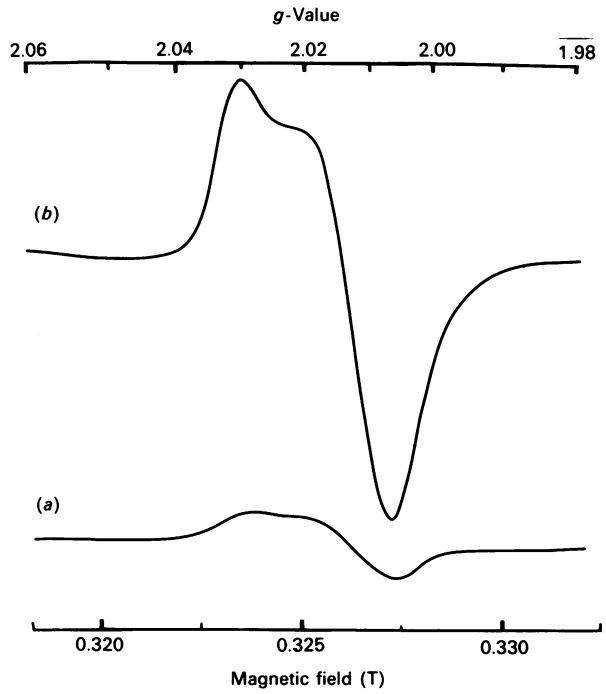

Fig. 5. First-derivative e.p.r. spectra of aconitase isolated from potato tuber mitochondria in the absence (*a*) or in presence (*b*) of 300 μM-H_2O_2

Aconitase was suspended to an appropriate protein concentration (100 μg/ml) in 10 mM-Tris/HCl buffer, pH 7.2. At 1 min after H_2O_2 addition, the sample was transferred to a quartz e.p.r. tube and rapidly frozen at 77 K. Conditions of measurement were: modulation amplitude, 0.1 mT; modulation frequency, 100 kHz; microwave frequency, 9250 MHz; microwave power, 0.5 mW; temperature, 10 K. Note that H_2O_2 induced a marked increase in the size of the e.p.r. signal of the Fe–S cluster present in aconitase.

unsuccessful. According to Beinert's group (Kennedy *et al.*, 1987), this full activation would involve the conversion of a $[3Fe–4S]^+$ cluster in the oxidized enzyme into the fully active $[4Fe–4S]^{2+}$ form.

It is evident from Fig. 5(*a*) that, in active plant mitochondrial aconitase, an e.p.r. signal of a 3Fe cluster was either barely detectable or not present. Experiments were therefore undertaken to oxidize this aconitase in a controlled fashion by using H_2O_2 in order to unmask a diamagnetic cluster.

Fig. 5(*b*) demonstrates that incubation of purified aconitase with small amounts of H_2O_2 caused the build up of a 3Fe cluster. The maximum enhancement was achieved after addition of 100 μM-H_2O_2 and was not increased further by up to 500 μM-H_2O_2. It should be noted that this e.p.r. spectrum resembled that of the oxidized mitochondrial matrix (Brouquisse *et al.*, 1986).

Fig. 6 indicates that inhibition of aconitase activity by H_2O_2 was a function of both the time and the concentration of the peroxide. Low concentrations of H_2O_2 increased the time required to block aconitase activity, whereas high concentrations of H_2O_2 decreased this time. At external H_2O_2 concentrations higher than 500 μM the inhibitory effect of H_2O_2 was virtually instantaneous. Again, the effectiveness of H_2O_2 in blocking aconitase activity was strongly reduced by citrate (result not shown). It was also noted that the increase in the signal at $g = 2.03$ did not coincide with the disappearance of aconitase activity. For example, exposure to 200 μM-H_2O_2 resulted in the total unmasking of the signal at $g = 2.03$ in less than the mixing time (approx. 10 s), whereas, under the same conditions, the inhibition of aconitase activity increased progressively (Fig. 6).

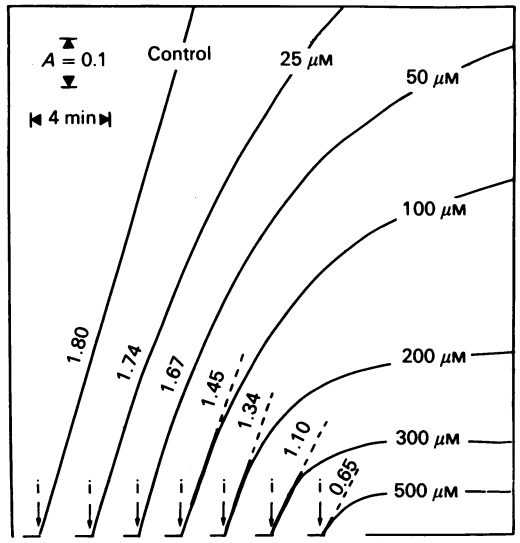

Fig. 6. Effect of H_2O_2 on the activity of aconitase isolated from potato tuber mitochondria

The preparation of aconitase (Trisacryl M DEAE pool; see Table 1) is described in the text. The standard reaction mixture (see the text) contained 30 μg of protein (final vol. 0.5 ml). Aconitase activity was assayed at 25 °C by monitoring the formation of *cis*-aconitate at 240 nm with a Kontron (Uvikon-810) spectrophotometer. H_2O_2 (25–500 μM) was added to the cuvette. The values along the traces refer to μmol of *cis*-aconitate formed/min per mg of protein upon adding 10 mM-isocitrate (i). Note that inhibition of aconitase activity by H_2O_2 was a function of both the time and the concentration of the peroxide.

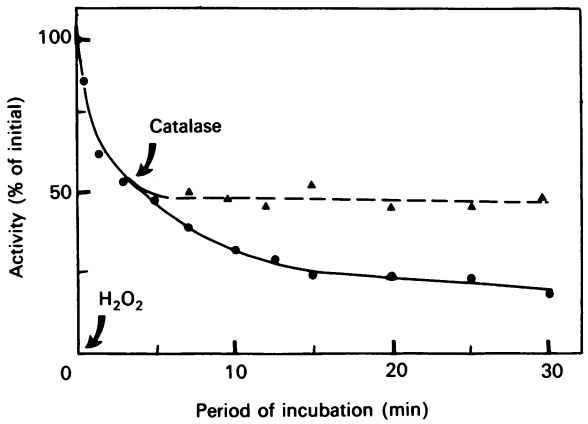

Fig. 7. Effect of catalase on the evolution of aconitase inhibition by H_2O_2

The preparation of aconitase is described in the text. The standard reaction mixture (see the text) contained 6 μg of aconitase/ml. At zero time 100 μM-H_2O_2 was added. At various times portion (0.5 ml) was taken for the measurement of aconitase activity by monitoring the formation of *cis*-aconitate at 240 nm with a Kontron (Uvikon-810) spectrophotometer upon addition of 10 mM-isocitrate. Where indicated, catalase (2.5 units) were added to the reaction mixture. Note that the evolution of aconitase inhibition by H_2O_2 is stopped (broken line) by the addition of a large excess of catalase to the reaction mixture. The initial aconitase activity was 2.4 μmol of *cis*-aconitate formed/min per mg of protein. The k_m value for isocitrate was 430 μM.

Interestingly, the evolution of aconitase inhibition was stopped by the addition to the medium of a large excess of catalase (Fig. 7), and attempts to re-activate H_2O_2-treated aconitase via either Fe^{2+} alone, or Fe^{2+} in combination with cysteine or thiosulphate, were unsuccessful.

DISCUSSION

The purification of aconitase from the lysed mitochondria isolated from potato tubers indicates that the e.p.r. signal typical of oxidized three Fe clusters with a peak at a g value of approx. 2.03 was almost undetectable. Likewise this signal was practically absent from the non-oxidized matrix of the potato tuber mitochondria. On the other hand, small amounts of H_2O_2 fully unmasked this signal, either in the matrix space or in highly purified mitochondrial aconitase. These results demonstrate that the signal typical of oxidized 3Fe clusters previously observed in the matrix space of plant mitochondria is attributable to oxidized aconitase (Brouquisse *et al.*, 1987). The unmasking of the oxidized 3Fe cluster, which is not representative of the status *in vivo*, leads to a progressive inactivation of aconitase in an irreversible manner and, ultimately, to a potent inhibition of citrate-dependent O_2 consumption by intact mitochondria. By contrast, we have previously reported the presence of an e.p.r. signal at $g = 2.03$ in intact potato tuber mitochondria (Brouquisse *et al.*, 1986). However, at that time we were not aware of the dramatic effects of H_2O_2 on aconitase, and, in order to maintain the mitochondrial preparation under aerobic conditions, we routinely included 50 μM-H_2O_2 (not indicated in that paper) in the mitochondrial suspension medium just before e.p.r. experiments. Since the State 3 rates of succinate, external NADH and 2-oxoglutarate oxidation were unaffected by H_2O_2 over the dose range 50–500 μM, we can conclude that aconitase present in the matrix space is the major intramitochondrial target for inactivation by H_2O_2.

During oxidative inactivation of the enzyme there was a lag between the change in the redox state of the Fe–S cluster, as monitored by e.p.r. changes, and the disappearance of activity. We interpreted this lag as evidence for either a slow conformational change in the protein after cluster oxidation, which then led to the change in activity, or to $HO^{\cdot}$ (hydroxyl) radicals generated by the Fenton reaction in close proximity to the active site. Indeed, it has been demonstrated that proteins which have been exposed to certain oxygen radicals exhibited altered primary, secondary and tertiary structures and can undergo spontaneous fragmentation (Davies, 1987). The fact that citrate prevents inhibition of aconitase activity by H_2O_2 strongly suggests that this peroxide interacts with the active site of the enzyme and probably with the unique ferrous atom of the cluster directly linked to citrate (Robbins & Stout, 1989). Much remains to be done in order to understand the whole cascade of events via oxidation of an iron–sulphur cluster ($[4Fe\text{–}4S]^{2+}$? $[3Fe\text{–}4S]^{0} + Fe^{2+}$?) that leads to inhibition of aconitase by H_2O_2. Interestingly, several iron proteins, including human haemoglobin (Puppo & Halliwell, 1988*a*) and leghaemoglobin from soybean (*Glycine max*) root nodules (Puppo & Halliwell, 1988*b*), are also degraded upon exposure to H_2O_2 with a release of iron ions. However, for these proteins a massive molar excess of H_2O_2 was required. In addition, incubation of spinach (*Spinacia oleracea*) ferredoxin with 200 μM-H_2O_2 for up to 2 h produced no significant change in the iron–sulphur cluster (Puppo & Halliwell, 1989). Likewise, complexes I and II of the respiratory chain contain 4Fe–4S clusters that do not seem to be affected at H_2O_2 concentrations which strongly inhibit aconitase activity. Indeed, these results indicated that succinate, isocitrate and oxoglutarate are effective respiratory substrates in H_2O_2-treated mitochondria.

In support of this observation, Zhang *et al.* (1990) demonstrated that H_2O_2 partially inactivated NADH dehydrogenase, NADH oxidase and cytochrome oxidase in bovine heart submitochondrial particles, but even 10% loss of these activities required at least 300 μM-H_2O_2.

Finally, the results reported here raise the problem of the presence of an aconitase in glyoxysomes. Indeed, considering the rapid inactivation of aconitase by H_2O_2 and the very poor affinity of catalase for its substrate [it is not possible to saturate the enzyme with H_2O_2 within the feasible concentration range (up to 5 M-H_2O_2) (Aebi, 1974)], it is difficult to understand how aconitase can operate in microbodies if there is a continuous production of H_2O_2 during the course of β-oxidation of fatty acids (Beevers, 1975). Interestingly, aconitase is the only enzyme related to fatty acid degradation in glyoxysomes that has not been purified and investigated extensively (Kindl, 1987). In fact, preliminary results carried out in our laboratory strongly suggest that glyoxysomes do not contain aconitase, and that the glyoxylate cycle requires a detour via the cytosol, which contains a powerful aconitase activity (Brouquisse *et al.*, 1987).

REFERENCES

Aebi, H. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.), vol. 2, pp. 673–682, Academic Press, New York

Beevers, H. (1975) in Recent Advances in the Chemistry and Biochemistry of Plant Lipids (Galliard, T. & Mercer, E. I., eds.), pp. 287–299, Academic Press, New York

Beinert, H. & Thompson, A. J. (1983) Arch. Biochem. Biophys. **222**, 333–361

Beinert, H., Ackrell, B. A. C., Kearney, E. B., & Singer, T. P. (1975) Eur. J. Biochem. **554**, 185–194

Brouquisse, R., Gaillard, J. & Douce, R. (1986) Plant Physiol. **81**, 247–252

Brouquisse, R., Nishimura, M., Gaillard, J. & Douce, R. (1987) Plant Physiol. **84**, 1402–1407

Chua, N. H. (1980) Methods Enzymol. **69**, 434–446

Davies, K. J. A. (1987) J. Biol. Chem. **262**, 9895–9901

Day, D. A. & Wiskich, J. T. (1984) Physiol. Vég. **22**, 241–261

Douce, R. & Neuberger, M. (1989) Annu. Rev. Plant Physiol. Plant Mol. Biol. **40**, 371–414

Douce, R., Bourguignon, J., Brouquisse, R. & Neuburger, M. (1987) Methods Enzymol. **148**, 403–420

Emptage, M. H. (1988) ACS Symp. Ser. **372**, 343–371

Fansler, B. & Lowenstein, J. M. (1969) Methods Enzymol. **13**, 26–30

Journet, E. P. & Douce, R. (1983) Plant Physiol. **72**, 802–808

Kennedy, M. C., Emptage, M. H., Dreyer, J. L. & Beinert, H. (1987) J. Biol. Chem. **258**, 11098–11105

Kennedy, M. C., Werst, M., Telser, J., Emptage, M. H., Beinert, H. & Hoffman, B. M. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 8854–8858

Kent, T. A., Dreyer, J. L., Kennedy, M. C., Huynh, B. H., Emptage, M. H., Beinert, H. & Münck, E. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 1096–1100

Kindl, H. (1987) in The Biochemistry of Plants: Lipids: Structure and Function (Stumpf, P. K., ed.), pp. 31–52, Academic Press, New York

Laemmli, U. K. (1970) Nature (London) **227**, 680–683

Ohnishi, T., Ingledew, J. W. & Shiraishi, S. (1976) Biochem. J. **153**, 39–48

Pickworth Glusker, J. (1971) Enzymes 3rd Ed. **5**, 413–439

Puppo, A. & Halliwell, B. (1988*a*) Biochem. J. **249**, 185–190

Puppo, A. & Halliwell, B. (1988*b*) Planta **173**, 405–411

Puppo, A. & Halliwell, B. (1989) Phytochemistry **28**, 3265–3270

Robbins, A. H. & Stout, C. D. (1989) Proteins: Structure, Function and Genetics **5**, 289–312

Rose, I. A. & O'Connel, E. L. (1967) J. Biol. Chem. **242**, 1870–1879

Ruzicka, F. J. & Beinert, H. (1974) Biochem. Biophys. Res. Commun. **58**, 556–563

Ryden, L., Ofverstedt, L. G., Beinert, H., Emptage, M. H. & Kennedy, M. C. (1984) J. Biol. Chem. **259**, 3141–3144

Zhang, Y., Marcillat, O., Giulivi, C., Ernster, L. & Davies, K. J. A. (1990) J. Biol. Chem. **265**, 16330–16336

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