Pulsed cytochrome *c* oxidase from the thermophilic bacterium PS3

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(Received 20 June 1984/Accepted 25 July 1984)

A *caa*₃-type terminal cytochrome *c* oxidase (EC 1.9.3.1) from the thermophilic bacterium PS3 containing three subunits showed conversion from resting into pulsed form. Upon pulsing (reduction and re-oxidation), the cytochrome *c* oxidase activity increased over 10-fold. This enhanced activity of the pulsed enzyme gradually decayed. Addition of phospholipids, necessary for the enzyme activity, did not affect this decay process. Small changes in the absorption spectrum were observed for the resting-into-pulsed transition and for H₂O₂ ligation to the pulsed enzyme. The e.p.r. spectrum of the resting enzyme was very similar to that of mitochondrial enzyme, but the transient *g* = 5, 1.78 and 1.69 set of e.p.r. signals, associated with the pulsed bovine heart oxidase, were not observed in the case of pulsed bacterium-PS3 enzyme.

Cytochrome *c* oxidase (EC 1.9.3.1) from bovine heart and *Neurospora crassa* are known to exist in 'resting' (as isolated) or 'pulsed' (reduced and re-oxidized) form in the fully oxidized state (Antonini *et al.*, 1977; Brunori *et al.*, 1983). This resting-into-pulsed transition enhances the catalytic activity (Antonini *et al.*, 1977; Brunori *et al.*, 1979) and reactivity towards the ligands of haem *a*₃ such as cyanide (Brittain & Greenwood, 1976; Shaw *et al.*, 1978a; Naqui *et al.*, 1984). Upon reduction and re-oxidation the Soret peak of the bovine heart cytochrome oxidase shifts from 418 nm to 427–428 nm, this being accompanied by a small enhancement of the α -peak. This form, long known as 'oxygenated' oxidase (Okunuki *et al.*, 1958; Oriei & Okunuki, 1963; Lemberg & Mansley, 1966), was equated with pulsed oxidase (Brunori *et al.*, 1981). Recently, we have shown that, upon reduction and re-oxidation, in the strict absence of H₂O₂, the 'pulsed' oxidase formed absorbs at 420 nm, as opposed to 428 nm for the 'oxygenated' oxidase. When H₂O₂ was added to the 420 nm-absorbing form, a peroxide complex was formed with a Soret peak at 428 nm, thus establishing clearly that 'oxygenated' oxidase is a peroxide-liganded form (Kumar *et al.*, 1984a,b). However, the precise structural and conformational differences between

resting and pulsed forms are not yet fully understood.

Bacterial cytochrome oxidases of *aa*₃-type are known to have much simpler subunit structures (Ludwig, 1980; Poole, 1983). The enzyme from the thermophilic bacterium PS3 is composed of three subunits (56000, 38000 and 22000 Da) and contains two haem *a* groups (cytochrome *aa*₃) and two copper atoms (Cu_a and Cu_a₃) with an intrinsic *c*-type cytochrome covalently bound to 38000 Da subunit (Sone & Yanagita, 1982).

The bacterium-PS3 cytochrome oxidase shows H⁺-pumping activity when reconstituted into phospholipid vesicles (Sone & Hinkle, 1982; Sone & Yanagita, 1984), binds typical ligands of cytochrome *a*₃ (N. Sone & P. Nicholls, unpublished work) and reacts with O₂ at low temperatures (Poole *et al.*, 1982; Sone *et al.*, 1984). These characteristics are similar to those of the mitochondrial cytochrome oxidase. It seemed relevant, therefore, to investigate whether the pulsed state could be induced in the bacterium-PS3 enzyme, and, if so, what are the properties of the pulsed bacterium-PS3 cytochrome oxidase.

Experimental

Bacterial cytochrome oxidase was purified from the thermophilic bacterium PS3 as described previously (Sone & Yanagita, 1982). The concentration of the enzyme was determined from

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dithionite-reduced-minus-oxidized (resting) spectra at 604–630 nm by using $\Delta\epsilon = 23.2 \text{ mM}^{-1} \cdot \text{cm}^{-1}$. Yeast cytochrome *c* from *Saccharomyces cerevisiae* (type VII) was purchased from Sigma Chemical Co. The concentration of cytochrome *c* was determined from reduced-minus-oxidized spectra at 550 nm by using $\Delta\epsilon = 21.2 \text{ mM}^{-1} \cdot \text{cm}^{-1}$. Soya-bean phospholipids (Asolectin) were purchased from Associated Concentrates (Woodside, NY, U.S.A.) and partially purified as described previously (Sone *et al.*, 1977). All other reagents were of best available grade and used without further purification.

Pulsed bacterium-PS3 cytochrome oxidase was prepared essentially as described by Kumar *et al.* (1984). In short, anaerobic bacterium-PS3 cytochrome oxidase was reduced by minimal dithionite in a Thunberg cuvette in the presence of nanomolar catalase. After spectroscopic determination that the reduction is complete, the reduced enzyme was mixed with air for 1 min. The peroxide derivative was prepared by adding 2 mM- H_2O_2 (final concentration) to the pulsed bacterium-PS3 oxidase, and is referred to below as 'pulsed-peroxide'. Where necessary, the samples were withdrawn from the cuvette and frozen in liquid N_2 in e.p.r. sample tubes in less than 30 s. When the activity tests were carried out, the pulsed bacterium-PS3 oxidase was diluted to a desired concentration after being re-oxidized and mixed rapidly (mixing time < 0.2 s) with an appropriate volume of reduced cytochrome *c*, and the change in absorbance was observed at 550 nm minus 540 nm.

All optical spectra were run on a Johnson Foundation dual-wavelength spectrophotometer, and e.p.r. spectra on a Varian E109 spectrometer equipped with an Air Products liquid-He cryostat.

Results and discussion

Cytochrome *c* oxidation rate

Most well-defined changes upon resting-into-pulsed transition are in kinetic properties. Under conditions of relatively high enzyme and low substrate concentrations, a much higher rate of cytochrome *c* oxidation following a strictly first-order kinetics occurred with the pulsed bacterium-PS3 oxidase, whereas a lower initial rate of oxidation, gradually accelerating and thus deviating from the first-order kinetics, was observed with the resting enzyme. The enhancement of activity was about 6-fold (without phospholipids) to about 12-fold (with phospholipids). A 3–5-fold enhancement of cytochrome *c* oxidase activity was also observed for the bovine heart oxidase under similar conditions, as described previously by Antonini *et al.* (1977) and Kumar *et al.* (1984a).

Fig. 1 shows the decay kinetics of the enhanced

Fig. 1. Activity decay of the pulsed bacterium-PS3 cytochrome *c* oxidase

Decay of the enhanced activity of the pulsed bacterium-PS3 oxidase in the presence (●; left-hand scale) and in the absence (○; right-hand scale) of phospholipid at 21°C is shown. The arrows indicate the points where the enzyme was pulsed. For activity measurements, a 0.5 ml portion of reduced yeast cytochrome *c* was mixed rapidly with 1.5 ml of bacterium-PS3 oxidase at different times after pulsing. Potassium phosphate buffer (40 mM), pH 7.4, containing 0.2% Triton X-100 was used. Where indicated (●), the enzyme solution contained 0.27 mg of phospholipids/ml. The final concentrations of bacterium-PS3 oxidase and cytochrome *c* were 0.3 μM and 5.4 μM respectively. The optical pathlength was 10 mm, and the reference wavelength was 700 nm.

activity of the pulsed bacterium-PS3 oxidase in the presence and in the absence of phospholipids. The decay is extremely slow, and the presence of phospholipid did not seem to affect the decay of the enhanced activity. However, the addition of phospholipids stimulated the enzymic activity severalfold, as reported previously (Sone & Yanagita, 1982). This decay in activity is not due to denaturation of the enzyme, since, upon pulsing once again after about 5 h of decay, almost all the initial high activity could be restored (Fig. 1). These experiments clearly show that the bacterial cytochrome oxidase also exhibits the resting-into-pulsed transition characterized by a large change in activity.

Optical spectra

Fig. 2(a) shows the absorption spectra of the resting, reduced, pulsed and pulsed-peroxide forms. Because of the presence of the intrinsic *c*-type cytochrome, changes in the Soret peak were not clear. In the α -band region, the broad peak around 596 nm found in the resting enzyme shifted towards the red and slightly intensified when pulsed. Upon peroxide addition, the shoulder

Fig. 2. Absolute and difference spectra of different states of bacterium-PS3 cytochrome *c* oxidase (a) Absolute optical spectra of resting (—), reduced (---), pulsed (.....) and pulsed-peroxide (— · —); the bacterium-PS3 oxidase concentration was 1.8 μM . (b) Corresponding difference spectra: pulsed minus resting (.....), pulsed-peroxide minus resting (—) and pulsed-peroxide minus pulsed (---). All other conditions were as indicated in Fig. 1 legend. Samples were prepared as described in the Experimental section.

around 424 nm slightly intensified, but the α -peak showed a slight decrease in intensity. These characteristics are more clearly seen in the difference spectra (Fig. 2b). The pulsed-minus-resting difference spectrum has peaks at 440 nm and 602 nm and a trough at 403 nm; upon formation of the complex with peroxide, the difference spectrum with respect to resting has peaks at similar positions, but the intensities of the peaks are slightly diminished, accompanied by an increase around 426 nm, which is clearly seen from the pulsed-peroxide-minus-pulsed difference spectrum. Although the spectral changes associated with the resting-into-pulsed and pulsed-into-pulsed-peroxide transitions are highly reproducible, the possibility that a part of the spectral changes might be an artifact (due to sluggishly reacting haem impurities) is not at present ruled out.

Results shown in Fig. 2 indicate that kinetically more active pulsed bacterium-PS3 oxidase shows similar types of spectral changes to those recently attributed to the pulsed state (Kumar *et al.*, 1984a) in the bovine heart oxidase, and this form readily binds H_2O_2 to produce pulsed-peroxide similar to the 'oxygenated oxidase' (428 nm form) of the bovine heart enzyme.

E.p.r. spectra

The e.p.r. spectra of bacterium-PS3 oxidase (Fig. 3) in the resting and pulsed states are very similar to the e.p.r. spectra published by Fee *et al.*

Fig. 3. E.p.r. spectra of the resting, pulsed and pulsed-peroxide forms of bacterium-PS3 cytochrome *c* oxidase. Wide-scan e.p.r. spectra of resting (a), pulsed (b) and pulsed-peroxide (c) forms of bacterium-PS3 cytochrome oxidase are shown. The samples were prepared as described in the Experimental section, and spectra were run at 7°K, with 100 kHz modulation frequencies, 16 G modulation amplitude, microwave frequency 9.28 GHz and power 2 mW.

(1980) for *Thermus thermophilus* cytochrome oxidase and bovine heart oxidase (van Gelder & Beinert, 1969).

Apart from the minor high-spin haem signal around $g = 6$ and the non-haem iron impurity signal around $g = 4.3$, the following resonances are clearly seen: a sharp low-spin haem signal around $g = 2.96$ and a higher-field component around $g = 2.22$ corresponding to a low-spin cytochrome *a*, a broad low-spin haem signal centred at $g = 3.4$ corresponding to *c*-type cytochrome, and a very strong signal typical of oxidase copper at $g = 1.99$. No significant differences are seen in going from the resting to the pulsed or pulsed-peroxide forms in the $g = 3.4$, 2.96 and 1.99 set of e.p.r. signals, suggesting that the cytochrome *a*, cytochrome *c* and the e.p.r.-visible copper sites of the bacterium-PS3 oxidase change very little in their structure during the resting-into-pulsed transition. This is very similar to the behaviour exhibited by these sites in the bovine heart enzyme (Shaw *et al.*, 1978a; Karlsson & Andréasson, 1981; Brudvig *et al.*, 1981; Kumar *et al.*, 1984b). Further, this lack of change in parameters for the e.p.r.-visible sites of bacterium-PS3 oxidase during the resting-into-pulsed transition suggests that most of the changes seen in optical spectra during the same transition are due to changes at the e.p.r.-invisible active site of the oxidase.

Two other points worth mentioning about the e.p.r. characteristics of the bacterium-PS3 oxidase are the following. (1) The minor absorption-type e.p.r. signal seen at $g = 2.43$ is eliminated during the resting-into-pulsed transition but re-appears somewhat more strongly in the pulsed-peroxide form. Such signals, with slightly different g -values, have been suggested to arise from hydroxy complexes of cytochromes (Shaw *et al.*, 1978b). (2) No signals similar to the $g = 5$, 1.78 and 1.69 set of signals observed for the recently re-oxidized bovine heart oxidase (Shaw *et al.*, 1978a) and shown by us to be associated with the pulsed form (Kumar *et al.*, 1984b) were observed under our conditions by us in any form of the bacterium-PS3 oxidase. This observation suggests that, despite the very similar kinetic properties, the active-site structure of the bacterium-PS3 enzyme may be different from that of the bovine heart enzyme. A comparison of the structural properties of the active sites of these two oxidases might be useful.

General discussion

Enhancement of the catalytic activity of cytochrome oxidases from bovine heart and thermophilic bacterium PS3 as a result of the resting-into-pulsed transition suggests at least two possibilities, as follows.

(1) Conformational change. It is possible that, upon reduction and re-oxidation, small but significant conformational changes occur in the active site, thus increasing the catalytic activity. In the case of the bovine heart enzyme, it has been suggested that the sulphur atom, bridging the Cu_{a_3} and Fe_{a_3} at the active site in the resting state, is bound to the Cu_{a_3} alone in the pulsed state, thus rendering it more active towards ligands, for example (Powers *et al.*, 1981; Chance *et al.*, 1983). A new set of e.p.r. signals at $g = 5$, 1.78 and 1.69 was also observed (Shaw *et al.*, 1978a) in the pulsed state, suggesting some changes in the active site. However, in the present study we did not observe any e.p.r. signal at $g = 5$, 1.78 and 1.69 upon pulsing the bacterium-PS3 oxidase. It thus seems clear that the active sites of the two oxidases are different, but at this point the specific difference and the reason for the difference is not clear. Whether the different subunit compositions of these two enzymes (from bacterium PS3 and bovine heart) has any effect on the haem environment is speculative at this time.

(2) Generation of the 'additional' active sites. It is not unlikely that 'additional' active sites may be generated as a result of reduction and re-oxidation, because some groups (during isolation) being attached to the active site in the resting state may render them inactive (or less active) in a process similar to the formation of haemichromes in haemoglobin (Di Iorio, 1981). Upon reduction, these groups may dissociate (if the dissociation constant is higher in the reduced form than in the oxidized form), thus creating an 'excess' of active sites. A similar type of possibility has been proposed for the activation of ascorbate oxidase (Gerwin *et al.*, 1974).

The bacterium-PS3 oxidase possesses only three subunits, as opposed to the other enzymes known to show the resting-into-pulsed transition, e.g. bovine heart oxidase [eight or more subunits (Capaldi, 1982; Kadenbach *et al.*, 1983)] and *Neurospora* oxidase [eight subunits (Weiss & Sebald, 1978)]. That the activation process occurs even in a relatively simple and 'primitive' oxidase is worthy of note. In conclusion, it may once again be noted that the data reported in the present paper show clearly that cytochrome oxidase from bacterium PS3 exhibits the resting-into-pulsed transition. The absorption spectra show small difference as a result of this transition. However, the transient e.p.r. signals at $g = 5$, 1.78 and 1.69 attributed to the more active pulsed oxidase from bovine heart did not appear in the case of the pulsed bacterium-PS3 oxidase.

This work was supported by National Institutes of Health Grants HL 31909 and HL 18708.

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