Reaction of *caa*₃-type terminal cytochrome oxidase from the thermophilic bacterium PS3 with oxygen and carbon monoxide at low temperatures

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(Received 15 March 1984/Accepted 3 May 1984)

Reaction of O₂ and CO with a *caa*₃-type terminal cytochrome oxidase (EC 1.9.3.1) from the thermophilic bacterium PS3 grown with high aeration was studied at low temperatures. The CO recombination at the temperature range studied (–50°C to –80°C) followed first-order kinetics with an activation energy of 29.3 kJ/mol (7.0 kcal/mol). In the presence of O₂ at –113°C the photolysed reduced form binds O₂ to form an 'oxy' intermediate similar to Compound A. At a higher temperature (–97°C) another intermediate, similar to Compound B, is formed as a result of electron transfer from the enzyme to the liganded O₂.

The terminal oxidase of a Gram-positive spore-forming thermophilic bacterium PS3 has been isolated as a copper-containing cytochrome *caa*₃ complex (ferrocycytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) composed of three subunits (Sone & Yanagita, 1982). This enzyme is heat-stable (Sone *et al.*, 1979) and shows H⁺-pumping activity (Sone & Hinkle, 1982; Sone & Yanagita, 1984). Cytochrome oxidases from bacteria such as *Paracoccus denitrificans* (Ludwig & Schatz, 1980) and *Thermus thermophilus* HB8 (Fee *et al.*, 1980; Honnami & Oshima, 1980) have also been reported to show H⁺-pumping activity (Solioz *et al.*, 1982; Sone *et al.* 1983; Yoshida & Fee, 1984) and to contain only two subunits (Ludwig & Schatz, 1980; Yoshida *et al.*, 1984). The spectral and functional properties of these bacterial *aa*₃-type cytochrome oxidases hitherto studied are very similar to those of the mitochondrial enzyme. Kinetic study of the *Paracoccus* enzyme with O₂ at room temperature supports this similarity (Ludwig & Gibson, 1981). It seems, however, necessary to investigate the intermediate steps of the reaction with O₂ before concluding that the reaction mechanism is also similar.

To elucidate the reaction mechanism of cytochrome oxidase with O₂, Chance *et al.* (1975a,b)

have introduced a low-temperature triple-trapping method. This method has been successfully employed to study bacterial respiratory systems also (for review see Poole, 1983). Poole *et al.* (1981) have studied the reaction of O₂ with membrane-bound bacterium PS3 respiratory system containing both cytochromes *aa*₃ and *o*. However, they did not report formation of spectrally distinctive intermediate compounds. A more-recent study by us on the effect of aeration condition of the thermophilic bacterium PS3 showed that the bacteria grown with strong aeration produced cytochrome *a*₃ and with low aeration cytochrome *o* as the terminal oxidase preferentially (Sone *et al.*, 1983).

In the present paper we report reactions of bacterial *caa*₃-type cytochrome oxidase purified from bacterium PS3 cells with O₂ and CO at low temperatures. To the best of our knowledge this is the first report showing that an *aa*₃-type bacterial terminal oxidase forms spectrally distinctive intermediates, similar to Compounds A and B of mitochondrial cytochrome oxidase (Chance *et al.*, 1975a, 1978), starting from the fully reduced enzyme.

Experimental

Bacterial cytochrome *c* oxidase (*caa*₃) was purified from the thermophilic bacterium PS3 as described previously (Sone & Yanagita, 1982). The concentration of the enzyme was determined from dithionite-reduced-minus-oxidized spectra at

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604nm minus 630nm by using $\Delta\epsilon = 23.2\text{mm}^{-1}\cdot\text{cm}^{-1}$. Bovine heart cytochrome oxidase was prepared by the modified Volpe-Caughey method (Yoshikawa *et al.*, 1977). All other reagents were of the best available grade and used without further purification.

A Johnson Foundation dual-wavelength spectrophotometer was used with a 0.2cm-pathlength cuvette. The temperature was maintained by a constant flow of N_2 gas cooled by liquid N_2 and controlled with a Johnson Foundation temperature controller within $\pm 0.5^\circ\text{C}$. Flash photolysis was achieved in the sample compartment of the spectrophotometer by using a xenon lamp coupled through an optical fibre.

Cytochrome oxidase was solubilized in the reaction mixture (1ml) containing 0.05M-potassium phosphate buffer, pH 7.4, 0.25% Tween 20 and ethylene glycol, 30% (v/v) for CO recombination and 25% (v/v) for reaction with O_2 , and reduced with 2mM-ascorbate and 0.03mM - *NNN'* - tetramethyl - *p* - phenylenediamine. (For CO-recombination studies a small amount of $\text{Na}_2\text{S}_2\text{O}_4$ was also added.) CO was bubbled through the reduced enzyme in the 0.2cm-pathlength cuvette for about 5 min at 0°C . Then the

cuvette was kept at room temperature for 5 min in the dark and cooled to -20°C for the addition of O_2 . O_2 was introduced by adding a 0.1 ml portion of air-saturated ethylene glycol (final concn. 34%, v/v) and gentle stirring. The oxygenated sample was then quickly frozen in a solid- CO_2 /ethanol bath (-78°C). The frozen sample was transferred to the sample-holder of the spectrophotometer and allowed to equilibrate at the desired temperature for several minutes. During this period the spectrophotometer slits were kept closed. Then CO was photolysed off with a xenon flash lamp.

Results and discussion

Photolysis of reduced CO-liganded bacterium PS3 enzyme and CO recombination

Fig. 1 shows a reduced ('post-flash')-minus-reduced CO-liganded ('pre-flash') difference spectrum in the absence of O_2 at -125°C . The spectrum is very similar to the usual reduced-minus-CO-liganded reduced difference spectrum of the bacterium PS3 enzyme at room temperature (Sone & Yanagita, 1982) except for a small blue-shift (about 1–2nm) of the peaks, which may be a temperature-induced effect. As shown in the inset

Fig. 1. Reduced-minus-CO-liganded reduced difference spectra of bacterium PS3 cytochrome oxidase at -125°C . The CO-liganded reduced form of the bacterium PS3 enzyme ($7\mu\text{M}$) was prepared as described in the Experimental section. The sample was photolysed with 70 flashes from a xenon lamp at -125°C . The 'post-flash' spectrum shown was recorded by taking the CO-liganded reduced form ('pre-flash' spectrum) as the reference. The broken line is the 'pre-flash'-minus-'pre-flash' spectrum, i.e. the baseline. The inset shows the change in absorbance in the α -region as a function of number of flashes given. The numbers in the inset show the number of flashes and the arrows show the direction of change in absorbance. The spectra were recorded by taking the 'pre-flash' as the reference.

of Fig. 1, seven or eight flashes were necessary to attain 50% dissociation of CO-liganded enzyme. On the other hand five or six flashes were necessary to attain the same percentage of dissociation for the bovine heart enzyme under identical conditions, suggesting that the quantum yield for the photodissociation of the bacterium PS3 enzyme is slightly lower than that of the bovine heart enzyme.

The CO-recombination kinetics of bovine heart cytochrome oxidase has been a matter of controversy. DeFonseka & Chance (1978, 1979) have found that there is no intermediate step between the fully photolysed and CO-bound states, whereas Clore & Chance (1978) have suggested that the process goes through at least one intermediate. Denis & Richaud (1982) reported that CO recombination is multi-exponential in plant mitochondria.

In the case of the bacterium PS3 enzyme the CO-recombination rate after photodissociation is extremely slow below -100°C . We measured the kinetics of CO recombination at -80°C to -50°C . In all cases the kinetics followed strictly a first-order reaction (Fig. 2a), and the rate constants were identical at all wavelength pairs studied (443–457 nm, 415–457 nm and 590–609 nm). It has also been shown that the CO-recombination rates are identical at these wavelengths and follow first-order kinetics at room temperature (Sone *et al.*, 1983). The activation energy determined from

an Arrhenius plot (Fig. 2b) gives a value of 29.3 kJ/mol (7.0 kcal/mol). This value is comparable with that for the bovine heart enzyme, which is reported to be 35.1 kJ/mol (8.4 kcal/mol) (Erecinska & Chance, 1972), and with that for the *Schizosaccharomyces* enzyme, namely 28.9 kJ/mol (6.9 kcal/mol) (Poole *et al.*, 1979).

Formation of intermediate compounds with O₂

When the CO-liganded reduced bacterium PS3 enzyme was photolysed in the presence of O₂ at -125°C to -130°C , no spectral change, other than that associated with photolytic removal of CO, was observed. This was because no binding of O₂ to the reduced enzyme occurred in that temperature range. The binding of O₂ to the reduced enzyme occurred at -113°C with a half-time of about 10 min.

This time-dependent formation of Compound A indicating O₂ binding to reduced enzyme is shown in Fig. 3. The difference spectrum with respect to the reduced state has a peak at 597 nm and trough at 607 nm, and these spectral characteristics are very similar to those of Compound A found for the bovine heart enzyme reported by Chance *et al.* (1975a,b, 1978). To ensure that this spectral change is not due to CO recombination, but due to O₂ liganding, the sample was flashed again. No change occurred, showing that this product is photo-stable. Thus this spectral change is due to O₂ binding only.

Fig. 2. Kinetics of CO recombination with bacterium PS3 cytochrome oxidase after photolysis

(a) A typical time course of CO recombination after the CO-liganded reduced sample has been photolysed (10–12 flashes with 1–2 s intervals) at -70°C . The kinetics was monitored at 443–457 nm. The results are shown in a semi-logarithmic plot. (b) Temperature-dependence of the first-order rate constants determined at different temperatures shown in an Arrhenius plot to estimate the activation energy. Error bars shown are standard deviations of at least three experiments.

Fig. 3. Formation of bacterium PS3 cytochrome oxidase Compound A at -113°C

The CO-liganded reduced form of bacterium PS3 cytochrome oxidase ($30\ \mu\text{M}$) was oxygenated at -20°C and frozen at -78°C . The sample was photolysed (50 flashes) at -113°C , and then the spectrum was recorded immediately. The sample was then scanned repetitively, the first spectrum ('post-flash' or fully reduced) being taken as the baseline. The numbers in the Figure show the time in minutes after the first scan, and the arrows show the direction of absorption change.

Compound A, formed at -113°C , was warmed up to -97°C and the kinetics were followed at this temperature, the first spectrum (within 1 min) recorded at -97°C being taken as the baseline. A different kind of spectral change from the formation of Compound A was observed, mainly around 606 nm, in a time-dependent fashion. Decrease of the absorption around 606 nm indicates that the electron transfer from reduced cytochrome aa_3 to bound O_2 occurs to form a second intermediate of the reaction with O_2 . The sample was then cooled back to -113°C , and the spectrum was recorded, the post-flash spectrum (the reduced form) being taken as the baseline, and is shown in Fig. 4. The spectral characteristics are very similar to those of Compound B of the bovine heart enzyme (Chance *et al.*, 1975a,b, 1978). When the temperature was raised to -80°C , further oxidation of both cytochromes a and c was observed. Although oxidation of cytochrome a ceased after 10 min, the oxidation of cytochrome c proceeded for 30 min (results not shown). These data indicate that electron flow from cytochrome a and cytochrome c occurred at -80°C . Evidently a pseudo steady state is established at -80°C , where electron transfer from cytochrome c to cytochrome aa_3 occurs at almost the same rate as that from cytochrome aa_3 to O_2 , leading to a constant degree of cytochrome a reduction.

When the temperature of the above sample, incubated for 30 min at -80°C , was raised to

Fig. 4. Formation of bacterium PS3 cytochrome oxidase Compound B at -97°C

Compound A prepared as indicated in Fig. 3 legend was warmed up to -97°C . The sample was repetitively scanned, the first spectrum at -97°C being taken as the reference. After 35 min (completion of reaction) the sample was cooled back to -113°C , and the spectrum shown was recorded, with the fully reduced spectrum at -113°C being taken as the reference.

-60°C , reduction of these cytochromes was observed. This is assigned to re-reduction of the enzyme by the excess of reducing agents present in the sample.

General discussion

The experiments described above clearly show that at low temperatures the bacterium PS3 enzyme forms Compounds A and B, as the bovine heart enzyme does. If we ignore the small difference in the spectra, the only difference was that Compound B formation and further oxidation of the bacterium PS3 enzyme occurred at a lower temperature than with the bovine heart enzyme. The reason for this difference is unclear at this moment, but it may be worth mentioning that the intrinsic covalently bound cytochrome *c* in bacterium PS3 cytochrome oxidase is functioning in a similar manner to cytochrome *c* bound to the high-affinity site of the mitochondrial enzyme (N. Sone & P. Nicholls, unpublished work), and may induce similar modulations of the enzyme properties to those found in the case of the bovine heart enzyme (Kumar & Chance, 1982).

Poole *et al.* (1981) have studied the reaction of bacterium PS3 cytochrome oxidase with O₂ using a membrane preparation containing both cytochromes *aa*₃ and *o*. They reported biphasic kinetics at -103°C, but did not describe formation of Compounds A and B. In the present study, it is possible to detect these intermediates by changing temperature carefully and using purified bacterium PS3 cytochrome oxidase from cells grown with strong aeration.

The preliminary experiment by us, cited in a review (Ludwig, 1980), with cytochrome oxidase prepared from bacterium PS3 cells grown with low aeration showed that cytochrome *o* instead of cytochrome *a*₃ was possibly functioning as a terminal O₂-reacting chromophore to oxidize cytochromes *a* and *c*. However, the present study shows that cytochrome *a*₃ is functioning similarly to the bovine heart enzyme, at least when the enzyme was prepared from strongly aerated bacterium PS3 cells. The possibility that cytochrome *o* reacts with cytochrome *a* in cells grown with low aeration is still open.

This work was supported by Grant HL31909 from the National Institutes of Health.

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