# Electron Transfer Kinetics of $caa_3$ Oxidase from *Bacillus stearothermophilus*: A Hypothesis for Thermophilicity

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ABSTRACT The $O_2$ reaction and the reverse electron transfer of the thermophilic $caa_3$ terminal oxidase of *Bacillus stearothermophilus* have been studied by laser flash-photolysis. The results show that both reactions, although studied at a temperature of 20°C, far from the optimal temperature of $>$ 60°C for $caa_3$, follow a kinetic behavior essentially identical to that observed with the electrostatic complex between mammalian cyt *c* and cyt *c* oxidase. In the $O_2$ reaction cyt *a* and cyt $a_3$ are very quickly oxidized; cyt *a* is then re-reduced *via* $Cu_A$, whereas cyt *c* oxidation is apparently rate-limited by the oxidation of $Cu_A$. Upon photodissociation of the mixed valence-CO $caa_3$, reverse electron transfer from the binuclear center to cyt $a^{3+}$ ($\tau_1 = 3$ $\mu$s) and $Cu_A^{2+}$ ($\tau_2 = 64$ $\mu$s) is observed, while cyt *c* is not reduced by any detectable level.

These results seem to rule out accounting for enzymatic thermophilicity by altered kinetics of intramolecular electron transfer involving the cyt center in the reduced configuration, which is very fast. On the basis of these results and previous data, we propose that thermophilicity involves an increased activation barrier for the reduction of cyt $a_3$-$Cu_B$ in the configuration typical of the oxidized site.

## INTRODUCTION

Extremophiles are microorganisms which grow optimally under very unusual environmental conditions of temperature, pH, and osmolarity. There has been a great deal of interest in these microorganisms in recent years because they express enzymes characterized by unusually high thermodynamic stability. Interestingly, the enzymes from thermophiles (the extremophiles growing optimally at temperatures above 50°C) are often almost completely inactive at room temperature. This property, called thermophilicity, is obviously distinct from thermostability.

Although terminal oxidases from several aerobic thermophiles have been purified (Fee et al., 1986), detailed information on the kinetics and mechanism of these enzymes compared to eukaryotic cytochrome (cyt) *c* oxidase is rather limited and the molecular basis of thermophilicity is essentially unknown. Multiple steps in the catalytic cycle of cyt *c* oxidase have been identified and the lifetime and spectroscopic properties of crucial intermediates characterized (Ferguson-Miller and Babcock, 1996; Malatesta et al., 1995; Einarsdóttir, 1995; Trumpower and Gennis, 1994; Babcock and Wikström, 1992; Han et al., 1990). The question we wish to address in this communication is the mechanistic basis of thermophilicity, i.e., those kinetic steps characterized by an increased activation barrier that account for the very low activity of thermophilic oxidases at mesophilic temperatures. Briefly, we have addressed, we believe for the first time, the problem of understanding thermophilicity of terminal oxidases.

To investigate this problem we have used as a model the $caa_3$-type cyt *c* oxidase purified from *Bacillus stearothermophilus*, a moderate thermophile. This oxidase, which has a heme-*c* containing domain fused to the $aa_3$ core, is both thermostable and thermophilic (De Vrij et al., 1989). Previous work (Giuffrè et al., 1996) demonstrated that at temperatures below 30°C, this enzyme displays some similarities with the electrostatic complex between mitochondrial cyt *c* oxidase and cyt *c* in the reaction with CO and $O_2$; because the latter is too fast for stopped-flow analysis, we were unable to identify a kinetic step which may account for thermophilicity.

The kinetics of the $O_2$ reaction and reverse electron transfer (eT) of $caa_3$, studied by laser flash-photolysis at 20°C, show that intramolecular eT processes starting from the reduced configuration are all very fast at 20°C, which seems to rule out that reaction steps involving reduced cyt $a_3$-$Cu_B$ center and reverse $a_3 \rightarrow a$ eT have unusually high activation barriers. We conclude that thermophilicity is controlled by the process involved in controlling the reduction of the oxidized cyt $a_3$-$Cu_B$ center, which was proposed to be slow and rate-limiting the turnover for beef heart oxidase (Verkhovsky et al., 1995; Brunori et al., 1997).

## MATERIALS AND METHODS

The $caa_3$ oxidase from *B. stearothermophilus* was purified as previously described (De Vrij et al., 1989) and stored at −70°C in 50 mM Tris, 300 mM NaCl, 1% octyl glucoside at pH 7.5 at a protein concentration of

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approximately 10 $\mu$M ($caa_3$). Ascorbate and *N,N,N′,N′*-tetramethyl-p-phenilenediamine (TMPD) were from Sigma Chemical Co. (St. Louis, MO).

The mixed valence-CO (MV-CO) enzyme was generated by anaerobic incubation of oxidized $caa_3$ under pure CO. Glucose (5 mM) and glucose oxidase (100 mU) were used to scavenge oxygen in the presence of catalase (7 $\mu$U). Formation of the MV-CO, followed by recording absorption spectra between 400 and 700 nm every 15 min, required approximately 1 h. The spectral features of the MV-CO revealed the presence of contaminating cyt *o* in $caa_3$ preparation (already seen by Giuffrè et al. (1996)), due to a portion of the enzyme incorporating heme *o* into the binuclear center under highly aerated growth conditions (Sone and Fujiwara, 1991).

## Kinetic experiments

Reverse eT was measured in a LKS.50 laser kinetic spectrophotometer (Applied Photophysics, Leatherhead, UK). The reaction was initiated by illuminating the MV-CO enzyme with a laser pulse (10 ns, 100 mW, 532 nm) using a SL282G Nd:YAG laser equipped with frequency doubling optics (Spectron Laser Systems, Rugby, UK). Absorption changes at a selected wavelength were collected with a Hewlett Packard 54520A digitizing oscilloscope. For flow-flash experiments, fully reduced CO-bound cyt $caa_3$ was mixed asymmetrically (1:5) with oxygen-saturated buffer using a modified SX.17MV sample handling unit (Applied Photophysics). The modification placed the stopped-flow cell at the end of a thermostatted umbilical, allowing it to reside in the optical train of the LKS.50 laser spectrophotometer. Despite the increase in the pathlength of the sample handling lines, we obtained a dead time of 6 ms for mixing a total volume of 400 $\mu$l. To ensure that mixing took place in an anaerobic environment, the drive syringes were surrounded by a solution saturated with dithionite. Timing of the mixing and subsequent laser flash was controlled by the software provided with the LKS.50 laser kinetic spectrophotometer by Applied Photophysics.

Data were analyzed by the software MATLAB (MathWorks, South Natick, MA) running on an Intel 486-based computer. Kinetic difference spectra were obtained according to the method of Henry and Hofrichter (1992), using singular value decomposition (SVD).

## RESULTS AND DISCUSSION

### The reaction with oxygen

When reduced $caa_3$ from *B. stearothermophilus* was mixed with $O_2$, cyt *c* and cyt $a_3$ were almost completely oxidized within 10 ms, whereas cyt *a* remained reduced (Giuffrè et al., 1996). To resolve this reaction we used the flow-flash approach, in which the $O_2$ reaction is initiated by pulsing the fully reduced CO-bound enzyme with a laser after mixing with $O_2$ (Gibson and Greenwood, 1963).

The time course at 445 nm (*a*-type hemes) indicated a very rapid decrease in absorbance ($k_1 = 4.6 \times 10^4\ s^{-1}$), followed by an increase ($k_2 = 9.6 \times 10^3\ s^{-1}$), and finally a slower decrease ($k_3 = 650\ s^{-1}$, Fig. 1). At 405 nm (*c*-type heme) we observed a biphasic absorption increase; the fast phase (30% of total amplitude) was almost synchronous with the first phase observed at 445 nm, whereas the slow phase (70% of total amplitude) proceeded at $k = 1.3 \times 10^3\ s^{-1}$.

Interpretation of these results is not easy, given the presence of several oxidation states of the four chromophores (cyt *a*, cyt $a_3$, cyt *c*, and contaminating cyt *o*, as discussed in Materials and Methods), whose optical contributions largely overlap in the Soret region. Nevertheless, the general pattern observed at 445 and 405 nm is very similar to that reported by Hill for both the electrostatic complex between mammalian cyt *c* and cyt $aa_3$ (Hill, 1991; Hill, 1994) and the $caa_3$ from *B. subtilis* (Hill, 1996), a mesophilic bacterium. On the basis of his data, Hill proposed a model in which electrons are sequentially transferred:

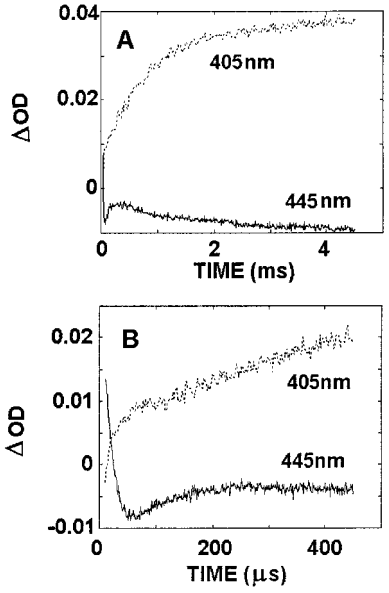


FIGURE 1 The reaction with oxygen. Fully reduced CO-bound $caa_3$ mixed in the stopped-flow with $O_2$ and subjected to a laser pulse at 20°C. [$caa_3$] = 2 $\mu$M; [$O_2$] = 1 mM. The fully reduced CO-bound enzyme was generated by reducing oxidase with ascorbate (5 mM) and TMPD (100 nM) under 1 atmosphere of CO. Absorption changes monitored at 445 nm and 405 nm over 4.5 ms (*A*) and 450 $\mu$s (*B*). Time courses were fitted to sum of exponentials.

$$\text{cyt } c \overset{1}{\leftrightarrow} Cu_A \overset{2}{\leftrightarrow} \text{cyt } a \overset{3}{\leftrightarrow} \text{cyt } a_3 - Cu_B \qquad (1)$$

The three phases we observed at 445 nm were therefore interpreted as an oxidative burst involving both cyt $a_3$ and cyt *a* (first phase), followed by a partial re-reduction of cyt *a* via $Cu_A$ (second phase) and the decay of the ferryl intermediate to a mixture of one-electron reduced species, mostly contributed by the species with the uneven electron residing on cyt *a* (third phase). This is consistent with our previous finding that when fully reduced $caa_3$ is pulsed with $O_2$, cyt *a* is only marginally oxidized at 10 ms after mixing (Giuffrè et al., 1996). Concerning the absorption changes at 405 nm, we assigned the rapid absorption increase to oxidation of the *a*-type hemes corresponding to the oxidative burst of the $aa_3$ core; the slower phase should be assigned largely to cyt *c* oxidation, proceeding at a rate somewhat faster (twofold) but possibly not inconsistent with the observed rate of cyt *a* oxidation ($k = 650\ s^{-1}$ in Fig. 1 *A*, 445 nm), given the limited resolution of the latter process.

These results demonstrate that the reaction of the $caa_3$ oxidase from *B. stearothermophilus* with $O_2$ at 20°C follows a kinetic behavior essentially identical to that observed with the electrostatic complex between beef heart cyt *c*

oxidase and horse heart cyt *c* (Hill, 1991). This finding reinforces the hypothesis that the $caa_3$ oxidase and the bovine enzyme share a common mechanism of eT to $O_2$ that is not altered by the covalently bound domain containing the *c*-type heme present in the bacterial enzyme. Thus, the relatively low activity observed at mesophilic temperatures cannot be accounted for by a slower $O_2$ reaction kinetics.

## Reverse electron transfer

When CO bound to MV-CO oxidase is photochemically dissociated by a laser pulse, the redox potential of the cyt $a_3$-$Cu_B$ binuclear center decreases and a fraction of the electrons residing on this site is back transferred to cyt *a* and $Cu_A$ with very fast relaxation rates (Boelens and Wever, 1979; Oliveberg and Malmström, 1991; Verkhovsky et al., 1992; Einarsdóttir et al., 1995). We have carried out these experiments with the $caa_3$ oxidase from *B. stearothermophilus*.

Upon laser illumination of the MV-CO derivative of $caa_3$, CO recombination was observed over the first 200 ms at different wavelengths (Fig. 2). The kinetic difference spectrum obtained by SVD (Fig. 2 *B*) was essentially identical to that observed upon CO binding to the fully reduced enzyme (Giuffrè et al., 1996) and showed the 417-nm peak that is characteristic of contaminating cyt *o*. This result indicates that, after photochemical dissociation, CO recombination to cyt $a_3$ and contaminating cyt *o* are synchronous, in agreement with expectations. The corresponding SVD analysis yields a CO recombination rate constant of $k' = 43\ s^{-1}$ at [CO] = 1 mM (Fig. 2 *A*, *inset*), a value somewhat smaller than that calculated from the bimolecular rate constant previously measured, $k = 7.8 \times 10^4\ M^{-1}\ s^{-1}$ (Giuffrè et al., 1996). This finding is expected on the basis of a partial oxidation of cyt $a_3$ due to reverse eT (Ädelroth et al., 1995).

Reverse $a_3 \rightarrow a$ eT was followed through absorption changes recorded over the first 180 $\mu$s after photolysis (Fig. 3 *A*). SVD analysis showed two kinetic phases; simultaneous fit of the first two U colums with two sequential decays (Fig. 3 *A*, *inset*) yields the corresponding kinetic difference spectra (Fig. 3 *B*). The larger absorption changes ($\tau = 3$ $\mu$s) correspond mainly to the oxidation of a fraction of cyt $a_3$ and synchronous reduction of an equivalent amount of cyt *a*. Due to the contribution of contaminating cyt *o* (see above), the kinetic difference spectrum displays a shoulder around 420 nm. On the other hand, the slower phase ($\tau = 64$ $\mu$s) is associated with the absorption changes typical of cyt *a* oxidation (solid spectrum in Fig. 3 *B*).

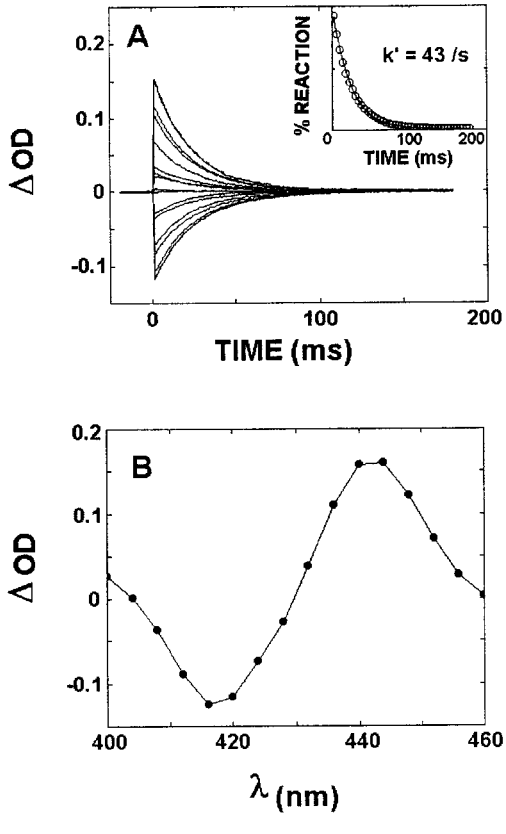


FIGURE 2 CO recombination to the MV-CO $caa_3$. (*A*) Millisecond absorption changes (monitored in the range from 400 to 460 nm) after laser photolysis of the MV-CO $caa_3$ (10 $\mu$M $caa_3$) in the presence of 1 mM CO at 20°C. This data set was analyzed by SVD. (*Inset*) Fit of the first U column to an exponential decay, with a rate constant $k' = 43\ s^{-1}$. (*B*) Kinetic difference spectrum observed on CO recombination to the MV $caa_3$ after CO photolysis. This spectrum is identical to the difference spectrum measured upon CO binding to fully reduced $caa_3$ (Giuffrè et al., 1996) and is partially contributed by a contaminating *o*-type heme.

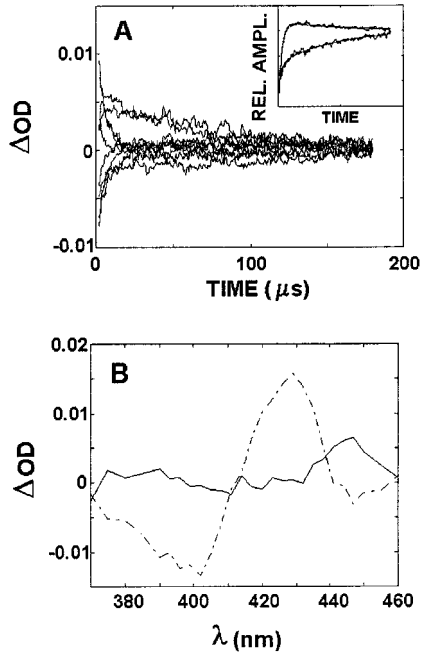


FIGURE 3 Reverse electron transfer. (*A*) Microsecond absorption changes (monitored in the range from 370–460 nm) after CO photolysis of the MV-CO enzyme (10 $\mu$M $caa_3$) at 20°C. SVD analysis clearly shows that two kinetic phases are present. (*Inset*) Fit of the first two U columns to a model involving two exponential decays in series: $\tau = 3$ $\mu$s (fast phase) and $\tau = 64$ $\mu$s (slow phase). (*B*) Kinetic difference spectra corresponding to the fast (*dashed spectrum*) and the slow (*solid spectrum*) phases.

These results are consistent with a reverse eT from the *cyt* $a_3$-$Cu_B$ binuclear center to cyt *a* ($\tau$ = 3 $\mu$s), followed by electron redistribution between cyt *a* and $Cu_A$ ($\tau$ = 64 $\mu$s). It is interesting to note that cyt *c* domain in the $caa_3$ oxidase was not significantly reduced over the same time range. This is consistent with the finding that reduction of cyt *a* is thermodynamically favored, as indicated by the higher reduction levels measured for cyt *a* at steady state (Giuffrè et al., 1996) and the equilibrium constant K = 3.5 in favor of cyt *a* reduction, estimated by following reduction of $caa_3$ during prolonged anaerobic incubation of the enzyme under CO (Bickar et al., 1984; data not shown).

The crucial observation in this experiment is that the reverse $a_3 \rightarrow a$ eT seen in the $caa_3$ oxidase of *B.stearothermophilus* at 20°C is very similar to that observed in the mammalian enzyme (Boelens and Wever, 1979; Oliveberg and Malmström, 1991; Verkhovsky et al., 1992; Einarsdóttir et al., 1995) in terms of both relaxation times and absorption changes.

## CONCLUSION

Analysis of the new results reported above, together with previous kinetic data obtained by stopped-flow analysis (Giuffrè et al., 1996), allows us to draw some conclusions about the step(s) at the root of thermophilicity in the $caa_3$ oxidase from *B. stearothermophilus*. As outlined in the Introduction, there is essentially no kinetic information suitable to account for the low activity of thermophilic oxidases at room temperature. This property is distinct from the more widely investigated thermostability of enzymes purified from extremophiles, and possibly more puzzling. Generally, it is to be expected that thermophilicity will be accounted for by an increased activation barrier for some (possibly just one) kinetic step in the overall reaction mechanism. We believe that our results allow us to propose a mechanistic hypothesis for the case of terminal oxidases.

If we refer to Eq. 1 (which, though oversimplified, realistically represents the kinetics of eT in cyt *c* oxidases), we see that *B. stearothermophilus* solved the problem of electron donation to $Cu_A$ (first step) by sythesizing an enzyme with the cyt *c* domain covalently attached to the oxidase moiety (De Vrij et al., 1989). This strategy allowed us to rule out substrate-enzyme complex formation and stability as a possible step in controlling thermophilicity. Moreover, we have evidence that at 20°C the *cyt c* $-$ $Cu_A$ eT is fast (Giuffrè et al., 1996) and we demonstrated above that at the same temperature, upon oxygen binding, cyt *c* becomes oxidized in the sub-ms range ($\tau \cong 500$ $\mu$s) after exposure to $O_2$. Interpretation of Fig. 1 also allows to state that re-equilibration of $Cu_A$ $-$ *cyt a* (step 2 in Eq. 1) is also very fast ($\tau$ = 64 $\mu$s) at 20°C and thus cannot account for thermophilicity. Finally, the results reported in Fig. 3 show that, when starting from a *cyt* $a_3$ $-$ $Cu_B$ site in a fully reduced configuration, internal eT between cyt *a* and *cyt* $a_3$ $-$ $Cu_B$ is also very fast ($\tau \cong 3$ $\mu$s). Indeed, this value is similar to that reported for beef heart cyt oxidase (Boelens and Wever, 1979; Oliveberg and Malmström, 1991; Verkhovsky et al., 1992; Einarsdóttir et al., 1995). Therefore these eT steps are all fast and similar to those reported for a number of mesophilic oxidases; hence, the origin of thermophilicity remains a puzzle.

We have previously suggested (Malatesta et al., 1990; Brunori et al., 1997) that in beef heart oxidase, the observed rate of (re)-reduction of the oxidized binuclear center is much slower than eT to the reduced site. Given the fixed distance between the donor (cyt *a*) and the acceptor (cyt $a_3$) and an unchanged driving force ($\Delta$G), it was suggested that such a difference could be attributed to a contribution from the reorganizational energy term $\lambda$ (Marcus and Sutin, 1985; Gray and Malmström, 1989; Brunori et al., 1994). This view is not universally accepted and Verkhovsky et al. (1995) proposed that, irrespective of the oxidation state of the binuclear center, the rate of heme-heme eT is fast but, because of thermodynamics, reduction of the binuclear center is slow and rate-limited by $H^+$ uptake. Although the merits of these alternative interpretations have to be assessed, our new data suggest that thermophilicity in the $caa_3$ oxidase from *B. stearothermophilus* might involve an increased energy barrier for the reduction of cyt $a_3$ starting from the oxidized binuclear center, which is an obligatory intermediate in the catalytic cycle (Babcock and Wikström, 1992). If reduction of cyt $a_3$ in the oxidized $caa_3$ is effectively slow and rate-limiting in turnover, as proposed for other terminal oxidases, we expect a considerably increased activation energy due to specific structural differences around the binuclear center of thermophilic oxidases.

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