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Triplet-state quenching in complexes between Zn–cytochrome *c* and cytochrome oxidase or its Cu_A domain

Peter Brzezinski [a], Mikael Sundahl [b], Pia Ädelroth [a], Michael T. Wilson [a,c], Bassam El-Agez [a,c], Pernilla Wittung [d], Bo G. Malmström [a,*]

[a] *Department of Biochemistry and Biophysics, Göteborg University, Medicinaregatan 9C, S-413 90 Göteborg, Sweden*
[b] *Department of Organic Chemistry, Chalmers University of Technology, S-412 96 Göteborg, Sweden*
[c] *Department of Chemistry and Biological Chemistry, University of Essex, Wivenhoe Park, Colchester CO4 3SQ, Essex, UK*
[d] *Department of Physical Chemistry, Chalmers University of Technology, S-412 96 Göteborg, Sweden*

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Abstract

The quenching of the triplet state of Zn–cytochrome *c* in electrostatic complexes with cytochrome oxidase and its soluble Cu_A domain has been studied by laser flash photolysis. The triplet state of free Zn–cytochrome *c* decayed with a rate of about 200 s^{-1}. With the oxidase, biphasic decay with rate constants of 2×10^5 and 2×10^3 s^{-1}, respectively, was observed. At high ionic strength ($I = 0.2$) the decay was the same as with free Zn–cytochrome *c*. The quenching was also eliminated by reduction of the oxidase. The decay rate in the complex with the Cu_A domain was 4×10^4 s^{-1}. The results are interpreted in terms of rapid electron transfer to Cu_A and a slower one to cytochrome *a*. No electron transfer products were detected, because the backward reaction is faster than the forward one. This can be explained by the high driving force (1.1 eV) for the forward electron transfer, taking the system into the inverted Marcus region. The distance in the electrostatic complex between cytochrome *c* and the electron acceptor, presumed to be Cu_A, is calculated to be 16 Å.

Keywords: Cytochrome oxidase; Zn–cytochrome *c*; Triplet state; Laser flash photolysis; Electron transfer

1. Introduction

Cytochrome *c* oxidase, the terminal enzyme of respiration in eukaryotes and some bacteria, contains three redox centers (see [1] for a review). Cytochrome *a* and Cu_A are the primary acceptors of electrons from cytochrome *c*. Electrons from the reduced primary acceptors are transferred intramolecularly to the bimetallic cytochrome a_3–Cu_B site, which is the dioxygen-reducing center. It has not been decided which of the two primary acceptors is the point of electron entry. The question cannot be answered by experiments on the stopped-flow time scale, since electron equilibration between cytochrome *a* and Cu_A has a rate constant close to 10^4 s^{-1} [2,3]. The reduction potential of cytochrome *a* is higher than that of Cu_A in the oxidized enzyme [4], so the first electron ends up on cytochrome *a* in the burst-phase of the reaction [5,6]. It can, however, have reached this site via Cu_A as the site of electron entry.

There are some indications that Cu_A is a more

* Corresponding author.

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facile electron acceptor than cytochrome *a*. The radical generated from 1-methylnicotinamide by pulse radiolysis reduces Cu_A very rapidly and preferentially [7]. In addition, the strongly reducing excited state of Tris(2,2′-bipyridyl)Ru(II) has been produced by flash photolysis in an electrostatic complex with the oxidase and found to reduce Cu_A in < 1 μs, whereas cytochrome *a* reduction is slower [8]. With photoactivated Ru(II)–cytochrome *c* at low ionic strength, Cu_A is reduced in a first-order process prior to cytochrome *a* reduction [9]. The objection to these experiments is, of course, that they involve unnatural electron donors, whose site of interaction may be different from that of cytochrome *c*. However, studies of the reaction of dioxygen with the fully reduced cytochrome *c*–cytochrome oxidase complex also indicate that Cu_A is the site accepting electrons from cytochrome *c* [10].

We have here approached the problem by studying the quenching of the triplet state of Zn–cytochrome *c* in its electrostatic complex with the oxidase formed at low ionic strength [11]. Zn–cytochrome *c* has been shown to bind at the same site as the native protein [12], and the quenching of its fluorescence has earlier been used to estimate donor–acceptor distances in the complex [13,14]. The triplet state, which lies 1.7 eV above the ground state [15], is formed with a 90% efficiency [16] and has a lifetime of 15 ms [17]. We found that in the complex it decays biphasically with decay times of about 5 μs and 500 μs, respectively. The quenching disappears on reduction of the oxidase and also on the addition of salt, which dissociates the complex. A rapid quenching with a decay time around 25 μs is observed in the electrostatic complex with the soluble Cu_A domain [18], which lacks the other redox centers; this effect also disappears at high ionic strength.

We interpret our results in terms of an electron-transfer quenching mechanism. Both Cu_A and cytochrome *a* can receive an electron from the triplet state, but the transfer to Cu_A is faster by a factor of about 100. We failed to detect any reduced forms of the oxidase or the Zn–porphyrin radical cation, but this is explained by the back reaction from the radical to the initial ground state being more rapid than the forward electron transfer from the triplet to Cu_A.

2. Materials and methods

2.1. Proteins

Bovine cytochrome oxidase was prepared by the method of Brandt et al. [19].

Zn–cytochrome *c* was prepared as described previously [14]. Its purity was checked by its optical spectrum. For making the Zn–cytochrome *c*–cytochrome oxidase complex, the buffer was changed to one with low ionic strength (25 mM Tris/acetate buffer, pH 7.8, containing 1% Tween 80) by gel filtration on a Sephadex G-25 column. Complex formation was demonstrated by Sephadex G-100 filtration [14]; it was also evidenced by the fact that a precipitate formed when the concentration was about 30 μM.

The soluble Cu_A domain of cytochrome oxidase subunit II was made as described in [18]. Bovine serum albumin was obtained from Sigma.

2.2. Spectroscopic methods

Optical spectra were recorded on a Cary 4 UV-visible spectrophotometer.

Two experimental systems were employed for the laser flash photolysis. One was a commercial system from Applied Photophysics. In this, a frequency-doubled Nd:YAG laser (Spectron Laser System, SL803G, 532 nm, 7 ns pulse width) was used for the excitation and a xenon arc lamp for the detection. The excitation and detection beams were at right angles to each other. A monochromator for the detection of light was placed in front of an R928 photomultiplier tube. The signal from the photomultiplier was fed into a digital oscilloscope (Philips PM 3323). The control of the timing sequence of the laser and shutters for the excitation and detection beams as well as the collection of data were all carried out on an Acorn Archimedes 440/1 computer.

The other system was of local design and has been described in detail elsewhere [20].

The measurements were made in closed 1-cm cuvettes, and the solution was flushed extensively with N_2 to eliminate O_2, which quenches the triplet state of Zn–cytochrome *c*. All kinetic traces were recorded at 22°C.

3. Results

The difference spectrum between the triplet and the ground state of Zn–cytochrome *c*, as recorded under our experimental conditions, is given in Fig. 1. This has been reported earlier [21] but only in the range 450–700 nm compared to 380–800 nm here. From the absorbances in Fig. 1, the concentration of Zn–cytochrome *c* and the molar absorbances [21], we conclude that 1/3 of the molecules in the detection path are excited. We deliberately used a low laser power in order not to photoinactivate the enzyme by the repeated laser flashes. This had the result that not all molecules reached the triplet state, but the same laser power was used in all experiments. The decay kinetics was measured at all wavelengths shown in Fig. 1, but most data were collected at 420 nm (Fig. 2), where there is the largest decrease in absorbance, or at 450 nm (Fig. 3), where there is a small absorbance increase.

The kinetics of triplet state decay with and without cytochrome oxidase is shown on our shortest time scale in Fig. 2. It can be seen that in the presence of a stoichiometric amount of oxidase, there is an initial rapid phase, which has a first-order rate constant of 2×10^5 s^{-1} and an amplitude 30% of the total absorbance change. The amplitude is less than maximal, when a substoichiometric amount of cytochrome oxidase is used, demonstrating that the quenching occurs in a complex with the oxidase. The rapid phase disappears on reduction of the oxidase. The small quenching still observed on this time scale is found also in the absence of oxidase, and it may be due to a small amount of porphyrin cytochrome *c* without Zn.

A slow phase of the triplet-state decay in the absence and presence of oxidase is illustrated in Fig. 3. The decay rate without oxidase is about 2×10^2 s^{-1}, which is slightly faster than reported in [17].

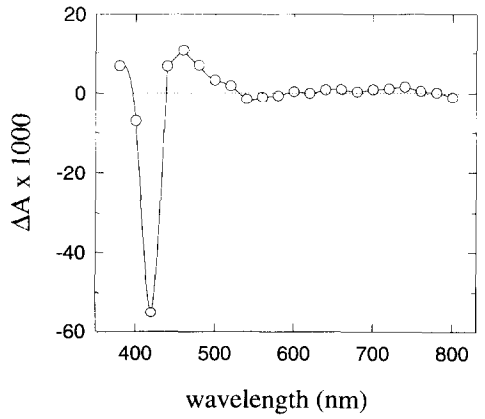

Fig. 1. The difference spectrum between triplet and ground state for Zn–cytochrome *c*. The concentration of Zn–cytochrome *c* was about 1.5 μM in 25 mM Tris/acetate, pH 7.8, with 1% Tween 80.

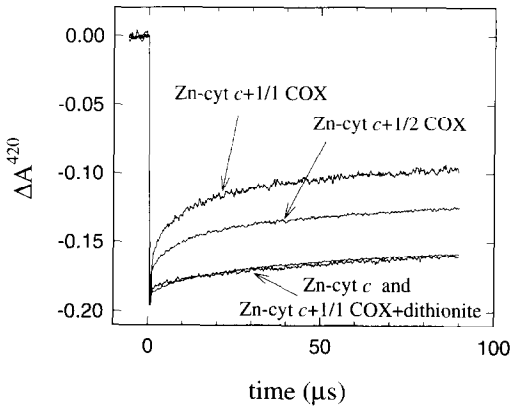

Fig. 2. The initial phase of the quenching of the triplet state of Zn–cytochrome *c* by cytochrome oxidase at low ionic strength with two different molar ratios measured at 420 nm. The effect of the reduction of the oxidase is also shown. The concentration of Zn–cytochrome *c* was 5 μM, and the buffer used was the same as in Fig. 1. COX in this and the following figures is an abbreviation for cytochrome oxidase.

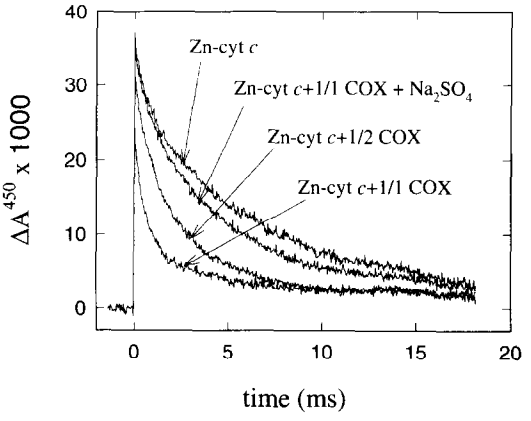

Fig. 3. The slow phase of the triplet state decay measured at 450 nm with conditions the same as in Fig. 2. The effect of 67 mM Na_2SO_4 ($I = 0.2$) is also shown.

This may be caused by traces of O_2 not removed by the flushing with N_2. In the complex, the decay has a rate constant of 2×10^3 s^{-1}, and again the amplitude titrates to this maximum value as the concentration ratio of Zn–cytochrome *c*/cytochrome oxidase is increased from 0.5 to 1. The quenching essentially disappears when the complex is dissociated by the addition of salt. Reduction eliminates this phase as well (data not shown). To exclude that a nonspecific binding to any negatively charged protein surface can lead to quenching, we made a complex between Zn–cytochrome *c* and serum albumin. In this case the triplet state decayed with the same rate as free Zn–cytochrome *c*, and there was no effect of an increase in ionic strength.

In a complex of Zn–cytochrome *c* with the soluble Cu_A domain, the triplet state was also quenched, as shown in Fig. 4. The decay rate in this case (4×10^4 s^{-1}) is intermediate between the slow and the rapid phase in the complex with the oxidase itself. There is also a slow component, which is due to unbound Zn–cytochrome *c*.

We were unable to detect any reduction of Cu_A at 830 nm or of cytochrome *a* at 445 or 605 nm. No formation of the Zn–cytochrome *c* radical cation could be observed at 675 nm [21].

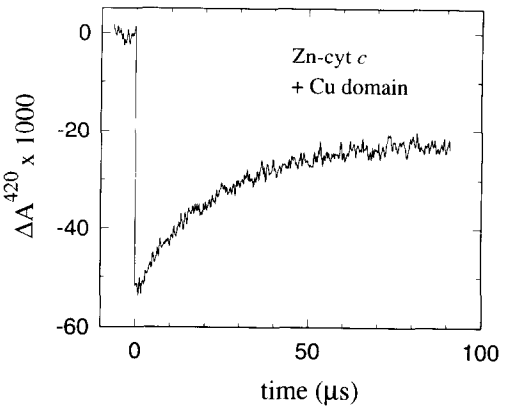

Fig. 4. The quenching of the triplet state measured at 420 nm in the complex between Zn–cytochrome *c* and the soluble Cu_A domain. The concentration of both proteins was 1.5 μM in 5 mM phosphate buffer, pH 7.4.

4. Discussion

The quenching of the triplet state of Zn–cytochrome *c* in its electrostatic complex with cytochrome oxidase has two kinetic phases (Figs. 2 and 3). Both phases achieve their maximum amplitudes in the 1:1 complex of the oxidase with cytochrome *c*. Thus, the two phases cannot be due to a heterogeneity of the enzyme, since then the phases would saturate at substoichiometric concentrations. Instead, there must be two modes of binding of the cytochrome with a slow (≥ 500 μs) equilibrium between them. If there were two quenching mechanisms in the same complex, or a rapid equilibrium between two complexes, then a complete quenching would occur with the rapid rate.

Regardless of the quenching mechanism, the rapid phase most likely involves Cu_A, because the quenching is only 5 times slower than the fast phase with the whole oxidase. The slower rate may be due to horse heart cytochrome *c* reacting less readily with the bacterial domain than with the more closely related bovine oxidase. This may also explain the weaker electrostatic interaction compared to the complex with the whole oxidase, as demonstrated by the decay component due to free Zn–cytochrome *c* (Fig. 4). There is evidence that cytochrome *c* binds to a negative patch on subunit II, which contains the Cu_A center [22]. It is possible that the stronger binding to the whole oxidase may instead be related to the finding that cytochrome *c* binds to subunit II at the interface of two oxidase molecules in a dimer structure [22]. Even if the soluble domain is made from a different species than the oxidase used, its Cu_A site appears to be identical, since it has the same spectroscopic properties and reduction potential as the native site, and it can be reduced by cytochrome *c* [18]. It should also be noted that in the electrostatic 1:1 complex with a Ru(II) compound, Cu_A and cytochrome *a* were both reduced directly, with Cu_A reduction being faster [8]. In addition, electrons from Ru–cytochrome *c* bound electrostatically to the oxidase enter Cu_A [9]. All these facts point to Cu_A as the source of the triplet-state quenching.

The rapid quenching is most likely due to electron transfer, as shown in the scheme in Fig. 5, rather than to energy transfer. The energy levels given for the different states are based on the known energetics of the system [16,21], with the reduction potential

of Cu_A being taken as 0.3 V (the exact value is 0.260 V [4]). The fact that the quenching disappears on reduction of the oxidase favors an electron-transfer quenching mechanism. If energy transfer were involved instead, it must be due to Cu_A, not to cytochrome *a*, because the spectral overlap with the latter would increase on reduction. Energy transfer to Cu_A does not appear possible, however, since the absorption bands of the soluble domain are at 480, 540 and 808 nm [18], and they do not overlap well with the Zn–cytochrome *c* phosphorescence spectrum [16].

If the quenching mechanism is electron transfer, then we have to explain why no electron transfer products could be observed in our experiments. An obvious possibility would be that $k_{b,ZnP} \gg k_{f,ZnP}$, as indicated in Fig. 5. With our signal-to-noise ratios, it can be estimated that it would be very difficult to detect any reduction of Cu_A smaller than 10%. Thus, we would not be able to observe formation of ZnP^+/Cu_A^+ with a backward rate 10 times faster than the forward rate. The larger driving force for the forward reaction may seem to contradict this explanation, but a real possibility is that the forward rate is in the Marcus inverted region [23] because the driving force is as large as 1.1 eV. This explanation is favored by the finding that $k_{f,c} \cong 1 \times 10^5\ s^{-1}$ with Ru–cytochrome *c* [9], i.e., half of the value with the triplet state of Zn–cytochrome *c*, despite the fact that the driving force ($\Delta G^0_{f,c}$) is more than 1 eV lower in this case. With this information, and with the rates and energetics given in Fig. 5, we can

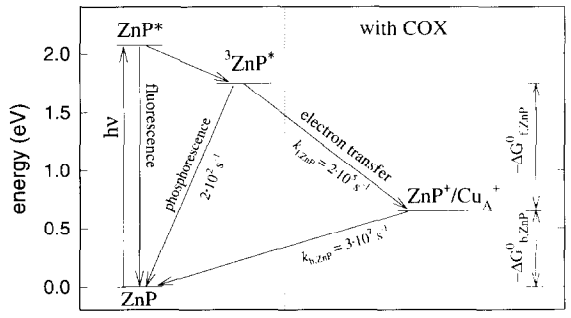

Fig. 5. Energy-level diagram for the singlet and triplet states of Zn–cytochrome *c* based on [16] and for the effect of cytochrome oxidase on the decay of the triplet state. The subscripts f,ZnP and b,ZnP refer to the forward and back electron transfer to cytochrome oxidase.

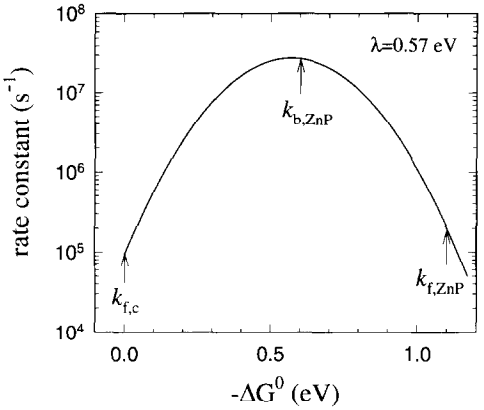

Fig. 6. Marcus plot of k_{ET} (on a log scale) as a function of increasing driving force ($-\Delta G^0$) with $\lambda = 0.57$ eV.

estimate the reorganization energy (λ), and hence $k_{b,ZnP}$, from a Marcus plot of log k_{ET} (the rate constant for electron transfer) versus $-\Delta G^0$ [23], as shown in Fig. 6. This treatment presupposes that the electron transfer is not gated in by conformational changes [24], as has been suggested for cytochrome *c* [25]. If there are only two conformations, one for the oxidized and one for the reduced protein, the Marcus formalism is still applicable, and the conformational change will be part of λ. It is only in a situation where there are more than two *stable* conformations (or, in other words, more than two energy minima) that the treatment breaks down. Later investigations [26–28] have shown, however, that there is no complication from conformational mobility in cytochrome *c*. In particular, λ calculated from the driving force dependence of the rate of electron transfer in the ruthenated protein [26] is the same as that derived from the temperature dependence [28], which is very strong evidence that the application of Marcus theory to cytochrome *c* is perfectly valid.

The curve in Fig. 6 is calculated on the basis of equations for the electron-transfer rate to a weakly coupled acceptor [23]:

$$k_{ET} = k_0 \exp(-\Delta G^*/k_B T) \quad (1)$$

$$\Delta G^* = \frac{\lambda}{4}\left(1 + \frac{\Delta G^0}{\lambda}\right)^2 \quad (2)$$

In Eqs. (1) and (2), k_0 is a constant which includes the electronic coupling, ΔG^* is the activa-

tion barrier, ΔG^0 the free energy difference between the donor and acceptor, λ the reorganization energy and k_B Boltzmann's constant. Assuming the same λ for the reaction with Zn–cytochrome *c* as for the native cytochrome *c*, we get:

$$\lambda = \frac{\Delta G^{0^2}_{f,ZnP} - \Delta G^{0^2}_{f,c}}{2\left[\left(\Delta G^0_{f,c} - \Delta G^0_{f,ZnP}\right) + 2k_B T \ln\left(\frac{k_{f,c}}{k_{f,ZnP}}\right)\right]} \quad (3)$$

With Eq. (3), we calculate a λ value of 0.57 eV. Thus, the rate constant for the back reaction ($k_{b,ZnP}$) is close to the maximum of the parabola, and the back reaction will be ≈ 100 times faster than the forward reaction. The relatively small reorganization energy also receives support by a comparison with some other systems. For example, in the case of electron transfer in a Ru–Zn–cytochrome *c*, a λ value of 1.2 eV was found [28], but this value decreased to 0.8 eV when the Ru ion was shielded from the solvent by a bulkier ligand [27]. If such a low λ can be found with an unnatural system with one of the electron-transfer centers on the surface of the molecule, then it is not unreasonable that an even smaller λ applies in a complex between two natural electron-transfer partners. For instance, for electron transfer between Cu_A and cytochrome *a*, λ has been estimated to be as small as 0.12 eV [29].

With the parameters in Figs. 5 and 6, we can calculate the distance between cytochrome *c* and the electron acceptor, here argued to be Cu_A. The constant k_0 in Eq. (1) depends on a frequency factor, ν_n, usually taken to be 10^{13} s^{-1}, and an electronic factor, Γ:

$$k_0 = \nu_n \Gamma = 10^{13}\Gamma \quad (4)$$

The electronic factor falls off exponentially with the distance, *d*, between the electron donor and acceptor, corrected for the van der Waals distance, d_0:

$$\Gamma = \exp[-\beta(d - d_0)] \quad (5)$$

where β is a constant. Other work with cytochrome *c* indicates that β for long-distance electron transfer in proteins is 1.0 $Å^{-1}$ [27]. Using this value and Eqs. (1–5), the distance between donor and acceptor is calculated to be 16 Å.

The slow phase probably involves interaction with cytochrome *a*. If the quenching is caused by energy transfer, then the distance between the two cytochromes must increase drastically on reduction. Thus, it appears likely that the quenching mechanism is electron transfer in this case as well. In the experiments with a complex of the oxidase with a Ru(II) compound [8], it was also found that both Cu_A and cytochrome *a* could be reduced directly. Reduction of cytochrome *a* has also been observed in oxidase molecules in which the Cu_A site has been removed [30]. In all cases, however, electron transfer is faster to Cu_A than to cytochrome *a*. The experiments reported here also suggest that Cu_A is the primary site of electron entry from cytochrome *c* into cytochrome oxidase.

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