Biochimica et Biophysica Acta, 682 (1982) 264–272
Elsevier Biomedical Press

BBA 41179

ELECTRON TRANSFER AFTER FLASH PHOTOLYSIS OF MIXED-VALENCE CARBOXYCYTOCHROME *c* OXIDASE

R. BOELENS, R. WEVER and B.F. VAN GELDER

Laboratory of Biochemistry, B.C.P. Jansen Institute, University of Amsterdam, P.O. Box 20151, 1000 HD Amsterdam (The Netherlands)

(Received May 5th, 1982)

Key words: Electron transfer; Flash photolysis; Cytochrome c oxidase; Redox center

The light-induced difference spectra of the fully reduced ($a^{2+}a_3^{2+}$-CO) complex and the mixed-valence carboxycytochrome *c* oxidase ($a^{3+}a_3^{2+}$-CO) during steady-state illumination and after flash photolysis showed marked differences. The differences appear to be due to electron transfer between the redox centres in the enzyme. The product of the absorbance coefficient and the quantum yield was found to be equal in both enzyme species, both when determined from the rates of photolysis and from the values of the dissociation constants of the cytochrome a_3^{2+}-CO complex. This would confirm that the spectral properties of cytochrome a_3 are not affected by the redox state of cytochrome *a* and Cu_A. When the absorbance changes after photolysis of cytochrome a_3^{2+}-CO with a laser flash were followed on a time scale from 1 μs to 1 s in the fully reduced carboxycytochrome *c* oxidase, only the CO recombination reaction was observed. However, in the mixed-valence enzyme an additional fast absorbance change ($k = 7 \cdot 10^3\ s^{-1}$) was detected. The kinetic difference spectrum of this fast change showed a peak at 415 nm and a trough at 445 nm, corresponding to oxidation of cytochrome a_3. Concomitantly, a decrease of the 830 nm band was observed due to reduction of Cu_A. This demonstrates that in the partially reduced enzyme a pathway is present between Cu_A and the cytochrome a_3-Cu_B pair, via which electrons are transferred rapidly.

Introduction

Cytochrome *c* oxidase is a mitochondrial enzyme that catalyses the electron transfer from cytochrome *c* to molecular oxygen. The enzyme contains four redox centres [1] which are all involved in the enzyme mechanism: cytochrome *a* accepts electrons from cytochrome *c*, Cu_A transfers the electrons to the cytochrome a_3-Cu_B pair, whereas cytochrome a_3 and Cu_B are involved in the binding and reduction of oxygen [2,3].

When both cytochrome a_3 and Cu_B are reduced, the enzyme binds CO [4–6]. There are two CO compounds of the enzyme [7]: a fully reduced complex ($a^{2+}\,Cu_A^{+}\,a_3^{2+}$-CO Cu_B^{+}), with cytochrome *a* and Cu_A reduced, and a mixed-valence complex ($a^{3+}Cu_A^{2+}\ a_3^{2+}$-CO Cu_B^{+}) with cytochrome *a* and Cu_A oxidised. These CO complexes are photodissociable [8] and a number of photolysis studies in the presence of O_2 have been carried out using optical and EPR spectroscopy [2,3,7,9–12]. A series of intermediates can be detected in which O_2 is increasingly reduced and the cytochrome a_3-Cu_B pair increasingly oxidised.

However, the photodissociation process itself was not studied in detail. It is known that the light-induced difference spectra of the fully reduced and mixed-valence CO-cytochrome *c* oxidase complexes are different [7]. These differences have been attributed to electron transfer [13] in the enzyme, coupling of the electron affinities of the redox sites in the enzyme [14], or to a spectral interaction between the haems of cytochrome *a*

and cytochrome a_3 [7]. Similar spectral haem-haem interactions were proposed from potentiometric titration studies [15]. EPR experiments, however, showed [16,17] that upon photodissociation of the mixed-valence CO complex and subsequent recombination with CO, the various redox centres in the enzyme could change their redox state according to:

$$X a_3^{2+} \cdot CO \overset{\text{light}}{\underset{CO}{\rightleftharpoons}} X a_3^{2+} \rightleftharpoons X^- a_3^{3+}$$

where X is cytochrome a or Cu_A. In the present paper such electron-transfer reactions were studied optically by a steady-state illumination technique and by laser photolysis. It could be shown that after flash photolysis of mixed-valence carboxycytochrome c oxidase rapid electron transfer occurred between cytochrome a_3 and Cu_A.

Materials and Methods

Cytochrome c oxidase was isolated from bovine heart as described previously [18,19]. The absorption coefficient of cytochrome c oxidase (reduced minus oxidised) was 24.0 $mM^{-1} \cdot cm^{-1}$ at 605 nm [20].

Chemicals were mainly from British Drug Houses (Analar Grade). CO (Matheson Gas Products) containing less than 10 ppm oxygen was freed from oxygen by passing it through an adsorption column filled with Oxisorb (Messer Griesheim). CO concentrations were calculated with Henry's Law and a solubility of 1 mM at a pressure of 100 kPa [21]. NADH, grade 2, was from Boehringer, the concentration was calculated with $\epsilon = 6.22$ $mM^{-1} \cdot cm^{-1}$ at 340 nm [22]. Phenazine methosulphate was from Sigma. The experiments were carried out in the absence of oxygen in anaerobic fluorescence Thunberg cells, which could be filled with CO of variable pressures. The samples were made anaerobic as described before [23]. The fully reduced cytochrome c oxidase-CO compound was prepared by adding CO to samples which were reduced by dithionite or excess NADH in the presence of phenazine methosulphate. The mixed-valence CO compound was prepared by incubation of oxidised cytochrome c oxidase under CO (60 kPa) at 20°C for 3 h, or through anaerobic reduction with NADH and phenazine methosulphate ($2e^-/aa_3$) as described previously [13].

The absorbance changes were studied with a single-beam spectrophotometer that could measure kinetic absorbance changes from 350 to 500 nm and from 750 to 900 nm. Photodissociation of cytochrome a_3^{2+}-CO was achieved by steady-state illumination with a 150 W xenon lamp filtered through an interference filter (590 ± 5 nm) or by a rhodamine dye laser (Phase-R) for fast kinetic studies. The lasing dye, rhodamine 590 (Exciton), was dissolved in methanol at a concentration of $5 \cdot 10^{-5}$ M. The laser had a maximum output at 580 nm and a 10% linewidth of 5 nm. The laser flash was 200 ns and had an intensity of about 1 J. The photomultiplier was protected against saturation by the laser light with optical filters BG 12 (350–500 nm) or RG 9 (750–900 nm) (Spindler and Hoyer). Because of the broad band output of the laser and the absence of suitable optical filters we were not able to suppress the saturation of the photomultiplier by the laser light to an acceptable level in the range from 550 to 650 nm. The anode of the photomultiplier (EMI 9559 or RCA IP 28) was connected to the ground with a resistor of 50Ω or 1 kΩ, and the signal amplified if necessary. The time response was controlled with a selected commercial LED and a Hewlett-Packard 3312A pulse generator ($\tau = 100$ ns with RCA IP 28, 50 Ω, gain 100 ×). The signal was recorded on a 5 MHz Transient recorder (Datalab 905), averaged with a microcomputer (Aim Rockwell) and stored in a Hewlett-Packard 2100B computer. In order to calculate the reaction rates, a computer programme was written in Fortran 4. The programme fitted the traces to exponential functions by a non-linear least-squares curve-fitting procedure as described before [24].

Results

Fig. 1 shows the light-induced absorbance changes after a laser flash in the fully reduced and mixed-valence CO complexes. The trough at 428 nm and the peak at 445 nm in the Soret region can be attributed to photodissociation of the cytochrome a_3^{2+}-CO complex and formation of cytochrome a_3^{2+}, respectively. The complexes show

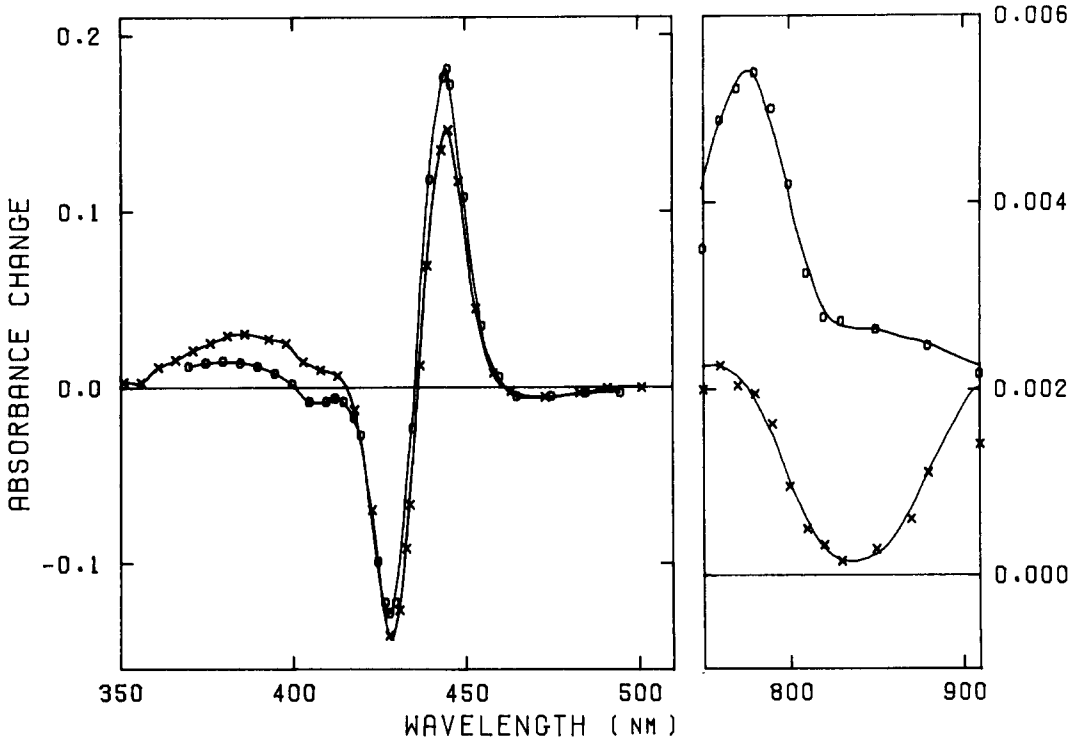

Fig. 1. Flash-induced absorbance changes in carboxycytochrome *c* oxidase. The absorbance change is the difference in the absorbance measured before and 1 ms after a laser flash. Conditions: 3 μM (Soret region) and 20 μM (near infrared) cytochrome *c* oxidase in 100 mM potassium phosphate (pH 7.4) and 1% Tween 80; $p_{CO} = 10$ kPa; temperature 20°C; O——O, fully reduced enzyme; ×——×, mixed-valence enzyme, prepared as described in Materials and Methods.

differences in the light-induced spectra: in the mixed-valence CO complex a smaller absorbance increase is observed at 445 nm and an increase at 415 nm. These differences are explained by oxidation of cytochrome a_3 in the mixed-valence enzyme after photodissociation in accordance with previous optical [13] and EPR experiments [16]. Under conditions of complete photodissociation the absorbance coefficients for the flash-induced changes are $\Delta\epsilon = 67$ $mM^{-1} \cdot cm^{-1}$ at 445 nm for the fully reduced enzyme and $\Delta\epsilon = 54$ $mM^{-1} \cdot cm^{-1}$ at 445 nm for the mixed-valence enzyme. These values are in close agreement with the results of Greenwood et al. [7], who found 64 and 56 $mM^{-1} \cdot cm^{-1}$, respectively. The difference in the light-induced spectra after a laser flash is less pronounced than that observed during steady-state illumination with a xenon lamp. In the flash photolytic experiment, the ratio $\Delta A_{445\ nm}/\Delta A_{428\ nm}$ decreases from 1.3 in the fully reduced CO complex to 1.1 in the mixed-valence CO enzyme. During steady-state illumination, however, this ratio changes from 1.3 to 0.85, in agreement with previous data [13]. The difference in results obtained by both techniques can be explained by a further oxidation of cytochrome a_3, which occurs between 1 ms after the flash and the time needed (50 ms) to reach the steady-state level during continuous illumination. This reaction could not be studied accurately in the Soret region, because of overlap with the CO recombination reaction.

In the near-infrared region also light-induced absorbance changes are detectable in the fully reduced CO complexes (Fig. 1). This indicates that cytochrome a_3 still has a small contribution ($\Delta\epsilon_{830} = 0.15$ $mM^{-1} \cdot cm^{-1}$) to the optical spectrum in the near infrared. Also a peak at 780 nm is observed, low in intensity, in the light-induced difference spectrum of the fully reduced CO complex. In the mixed-valence enzyme the light-in-

duced absorbance differences are much smaller than in the fully reduced enzyme. In addition, a trough is formed absorbing at 830 nm. This suggests that Cu_A^{2+}, which absorbs at this wavelength [23], is reduced after the laser flash. Using the absorbance coefficient (reduced minus oxidised) of 2.3 $mM^{-1} \cdot cm^{-1}$ at 830 nm for Cu_A^{2+} and the difference in the light-induced absorbance spectra ($\Delta\epsilon_{830} = 0.13$ $mM^{-1} \cdot cm^{-1}$), it is possible to calculate a fractional reduction of 0.05 during saturating light flashes.

Greenwood et al. [7] obtained similar difference spectra in the Soret region by extrapolating the light-induced absorbance changes to $t = 0$ after a flash. They propose, however, that in both complexes the spectral properties of cytochrome a_3 are different. To settle this point, the photodissociation reaction during steady-state illumination was studied in more detail and the product of the absorbance coefficient (ϵ) and quantum yield (ϕ) of both CO complexes was determined. These products were obtained from the rates at which equilibrium was established after photolysis and from the values of the equilibrium dissociation constants for CO as a function of the light intensity. The rates (k) and dissociation constants (K_d) obey the following equations [25,26]:

$$k = k_{on} \cdot [CO] + k_{off} + (\epsilon\phi I_0) \cdot \frac{I}{I_0} \quad (1)$$

$$K_d = K_{d,dark} + \left(\frac{\epsilon\phi I_0}{k_{on}}\right) \cdot \frac{I}{I_o} \quad (2)$$

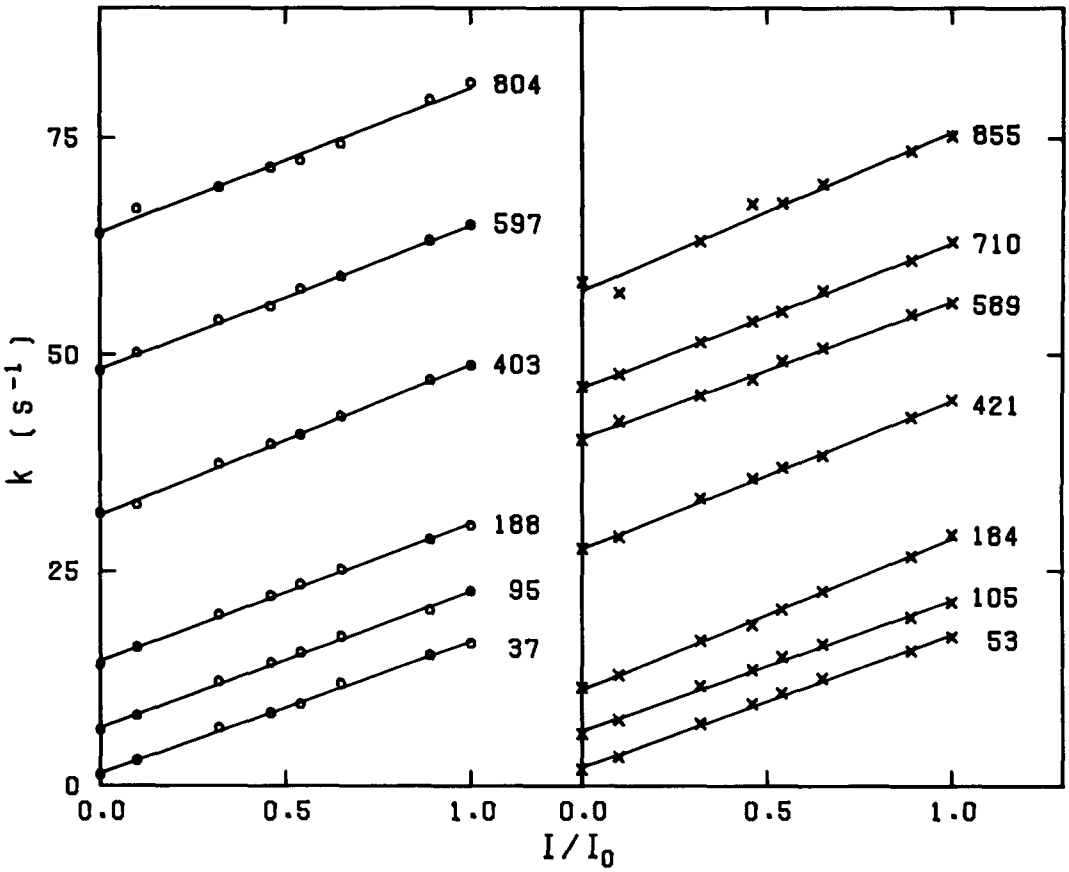

Fig. 2. Rates of photolysis of cytochrome a_3^{2+}-CO as a function of the light intensity at different concentrations of CO. Illumination was achieved by continuous irradiation with a xenon lamp. Conditions as in Fig. 1. The CO concentrations are as indicated in the figure in μM. The rates were measured at a wavelength of 445 nm. O——O, fully reduced enzyme; x——x, mixed-valence enzyme.

where k_{on} and k_{off} are the association and dissociation rate constants of the equilibrium

$$Fe^{2+} + CO \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} Fe^{2+} \cdot CO$$

$K_{d,dark}$ is the equilibrium dissociation constant in the dark; ϵ is the absorbance coefficient of cytochrome a_3^{2+}-CO at the excitation wavelength (590 nm); ϕ is the quantum yield of the photodissociation process, i.e., the ratio of the number of photodissociated molecules and the number of excited molecules; I_0 is the light intensity of the xenon lamp at 590 nm; I/I_0 is the transmission of the absorbance filters which were used to change the light intensity.

Fig. 2 shows the rates of photolysis of the cytochrome a_3^{2+}-CO complex as a function of the light intensity at different concentrations of CO. It is clear from the straight lines that Eqn. 1 is obeyed.

By measuring the absorbance at 445 nm at each CO concentration (not shown), it was possible to construct CO-binding curves at several light intensities. Under all conditions, CO binds non-cooperatively to reduced cytochrome a_3 in the fully reduced complex in line with the results in Ref. 13, as well as in the mixed-valence complex. From these binding curves the apparent dissociation constants could be determined. Fig. 3 shows that these dissociation constants increase linearly with light intensity as predicted by Eqn. 2. It is clear that the value of the dissociation constant for the fully reduced enzyme is lower than that for the mixed-valence enzyme at a certain light intensity.

Table I summarises the results of the kinetic and equilibrium studies. It is obvious that the product of absorbance coefficient and quantum yield, $\epsilon\phi$, is unaffected by the redox state of cytochrome *a* and Cu_A. Though it is conceivable that changes in ϵ and ϕ would just compensate each other, it is more likely that both ϵ and ϕ at 590 nm are the same for both complexes. Therefore, we conclude that the spectral properties of cytochrome a_3 are not affected by the redox state of cytochrome *a* and Cu_A, in line with the conclusions in Refs. 13 and 23. The fully reduced enzyme and the mixed-valence enzyme have different association rate constants ($7.84 \cdot 10^{-4}$ and $6.64 \cdot 10^{-4}$

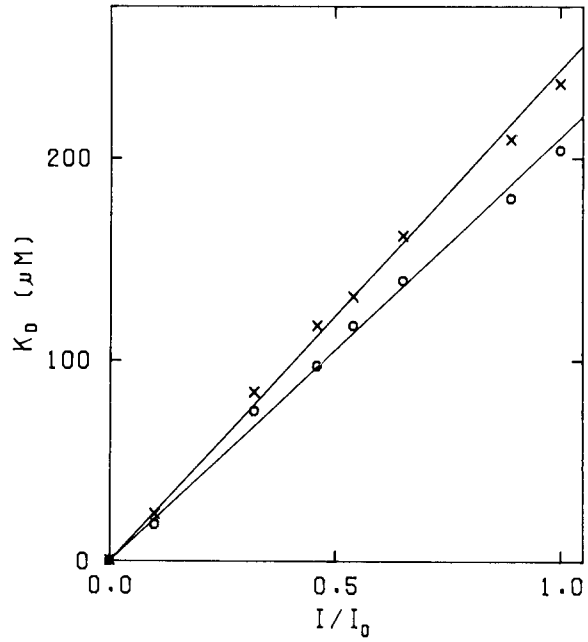

Fig. 3. Dissociation constants of the cytochrome a_3^{2+}-CO complex as a function of the light intensity. Conditions as in Fig. 2. O——O, fully reduced enzyme; x——x, mixed-valence enzyme.

TABLE I

KINETIC AND EQUILIBRIUM CONSTANTS OF THE PHOTODISSOCIATION AND RECOMBINATION REACTIONS OF CYTOCHROME a_3^{2+}-CO IN FULLY REDUCED AND MIXED-VALENCE CYTOCHROME *c* OXIDASE

	Kinetics			Equilibrium		
	k_{on} ($M^{-1} \cdot s^{-1}$)	k_{off} (s^{-1})	$\epsilon\phi I_0$ (s^{-1})	$K_{d,dark}$ (μM)	$\epsilon\phi I_0/k_{on}$ (μM)	$\epsilon\phi I_0$ s^{-1})
Fully reduced	$7.84 \cdot 10^4$	0.03	16.5	0.33	209	16.4
Mixed-valence	$6.64 \cdot 10^4$	0.04	16.4	0.6	241	16.0

$M^{-1} \cdot s^{-1}$, respectively) and dissociation rate constants (0.03 vs. 0.04 s^{-1}). The dissociation rate constants were measured by exchanging CO for O_2 at low CO concentrations. This was achieved by opening the Thunberg cell and mixing the solution thoroughly with air. The rate of oxidation of the reduced cytochrome *c* oxidase was taken as a measure of the rate of dissociation of CO. Unfortunately, by this method the rates could not be determined very accurately. Table I also gives the equilibrium dissociation constants for both complexes, as obtained from the binding curves in the dark. The dissociation constants of the fully reduced complex (0.33 μM) and mixed-valence complex (0.6 μM) are in good agreement with the values calculated from the kinetic data (0.38 and 0.6 μM, respectively). As will be discussed later, at least part of the difference in binding between both complexes is due to a statistical factor.

Since no optical interaction between cytochrome a_3 and the other redox sites exists, the absorbance differences observed in Fig. 1 upon illumination of both CO complexes must be explained by an electron-transfer reaction which occurs after photolysis of the mixed-valence CO complex. It is possible to follow the electron transfer by studying the absorbance changes induced by a short laser flash in the Soret region and at 820 nm. Fig. 4 shows the absorbance changes after the flash in the mixed-valence CO complex and in the fully reduced complex. First, there is an immediate absorbance increase at 445 nm as well as

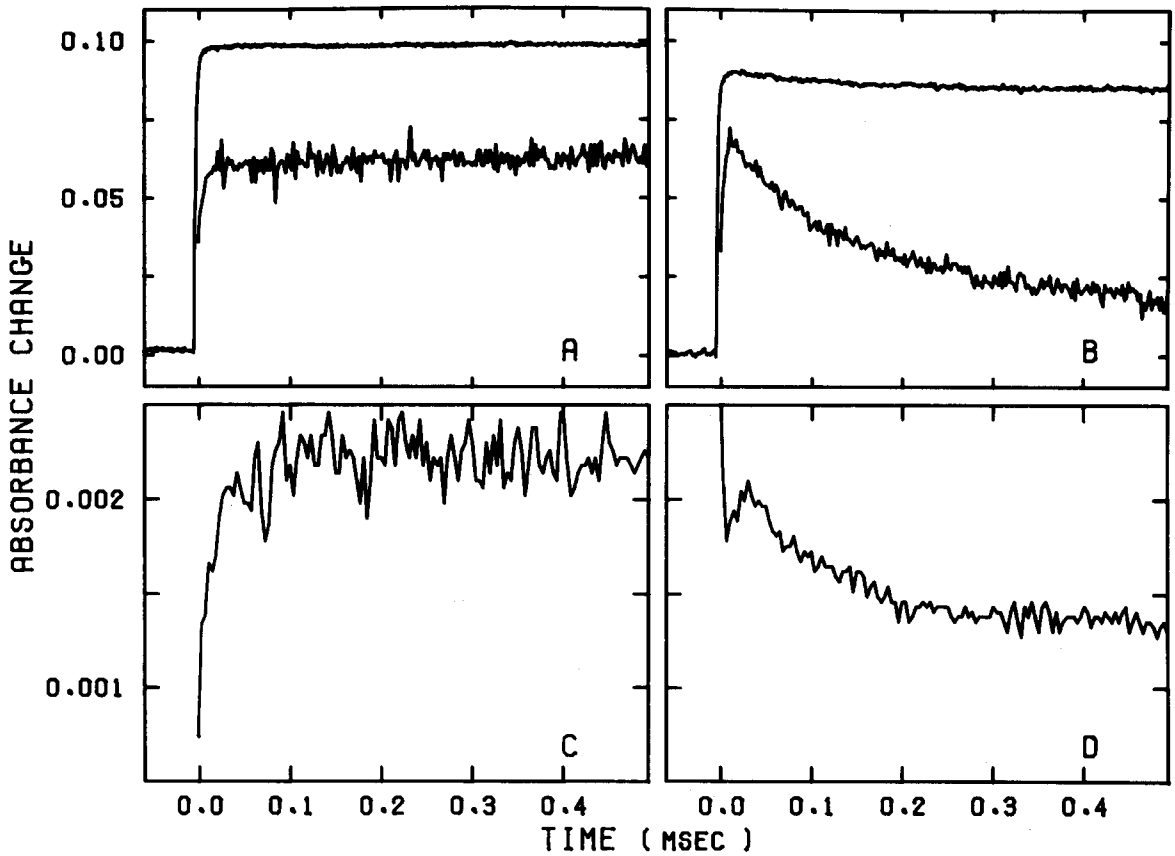

Fig. 4. Absorbance changes at 445 and 820 nm after flash photolysis of carboxycytochrome *c* oxidase. Conditions: 1.5 μM (A,B) and 8 μM (C,D) cytochrome *c* oxidase, 100 mM potassium phosphate and 1% Tween 80; p_{CO} = 10 kPa; temperature 20°C; A, fully reduced enzyme at 445 nm (inset, 10× more sensitive absorbance scale); B, mixed-valence enzyme at 445 nm (inset, 10× more sensitive absorbance scale); C, fully reduced enzyme at 820 nm; D, mixed-valence enzyme at 820 nm. The transients were averaged 128 times.

at 820 nm due to the formation of cytochrome a_3^{2+}. In mixed-valence CO-cytochrome *c* oxidase this increase in absorbance is followed by a fast decrease at 445 nm and at 820 nm due to the oxidation of cytochrome a_3^{2+} and the reduction of Cu_A^{2+}, respectively. These fast absorbance changes were followed by the CO recombination reaction which was slower. The rate of the fast reaction was independent of the CO concentration, although at higher CO concentrations the recombination reaction interfered with the fast redox change. The sequence of the light-induced reaction was reversible and the process could be repeated many times.

Both fast transient of the electron tranfer fit an exponential curve with a rate constant of $7.0 \cdot 10^3$ s^{-1}. In saturating laser flashes $\Delta\epsilon$ at 445 nm for this fast reaction was $-5.7\ mM^{-1} \cdot cm^{-1}$, whereas at 820 nm $\Delta\epsilon$ was $-0.12\ mM^{-1} \cdot cm^{-1}$. Since both cytochromes contribute about equally to the reduced-minus-oxidised spectrum of cytochrome *c* oxidase at 445 nm, as ligand-binding experiments [27] and redox titrations [14] have suggested, the $\Delta\epsilon$ (reduced minus oxidized) for cytochrome a_3 at 445 nm is 80 $mM^{-1} \cdot cm^{-1}$. Thus, the absorbance decrease at 445 nm corresponds to a partial oxidation of cytochrome a_3 of 0.07. On the other hand, using a $\Delta\epsilon$ (reduced minus oxidised) of 2.3 $mM^{-1} \cdot cm^{-1}$ for Cu_A [23] a fractional reduction of this component of 0.05 is observed.

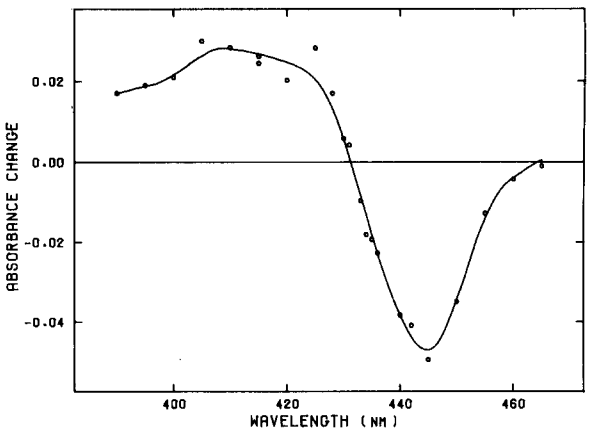

Fig. 5. Kinetic difference spectrum of the oxidation of cytochrome a_3 after flash photolysis of mixed-valence carboxycytochrome *c* oxidase. Conditions: 8 μM cytochrome *c* oxidase, 1% Tween 80 and 100 mM potassium phosphate (pH 7.4); temperature 20°C; $p_{CO} = 10$ kPa. The absorbances are the extent of the transients measured at several wavelengths, after flash photolysis of the mixed-valence enzyme. The extents were obtained by curve fitting to these transients and extrapolating to $t = 0$. The transients were averaged 8–64 times, depending on the signal-to-noise ratio.

Fig. 5 shows the extent of the fast absorbance changes of this transfer, plotted as a function of wavelength in the Soret region. The absorbance decrease at 445 nm and increase at 415 nm confirm that oxidation of cytochrome a_3^{2+} does occur. Similar difference spectra for cytochrome a_3 were obtained from ligand-binding experiments [27] and the reaction of reduced cytochrome *c* oxidase with oxygen [2]. By measuring the absorbance at 445 nm before and 1 ms after the laser flash and by extrapolating the absorbance changes at 445 nm to $t = 0$, an absorbance coefficient for the light-induced change of $\Delta\epsilon = 63 \pm 2\ mM^{-1} \cdot cm^{-1}$ at 445 nm can be calculated for the mixed-valence enzyme. This is very close to the absorbance change observed in the fully reduced enzyme ($\Delta\epsilon = 67\ mM^{-1} \cdot cm^{-1}$). Within the accuracy of the experiments and the time resolution of the instrument it cannot be decided whether or not there is still a small spectral difference in the light-induced absorbance changes in the mixed-valence and the fully reduced enzyme. It is, however, clear that a large part of the observed differences in Fig. 1 is due to the redox reaction as observed in Fig. 5.

Discussion

As shown here, the CO binding and dissociation reactions in cytochrome *c* oxidase are similar to those observed in other haemoproteins [25,26]. The product of absorbance coefficient and quantum yield was the same for the fully reduced and the mixed-valence CO complexes of cytochrome *c* oxidase. This suggests that cytochrome a_3 in both complexes has the same optical properties. This result strengthens the idea that the optical properties of one redox centre in the enzyme, cytochrome a_3, are not strongly affected by the redox state of the other two redox sites (Cu_A and cytochrome *a*). This finding is important, since from optical redox titration studies it was concluded that an interaction exists between the redox sites. However, it could not be decided whether this interaction had an optical origin, or that the electron affinities of the redox sites were interdependent [28]. From our

results, as well as from MCD studies of cytochrome *c* oxidase [29,30], it was concluded that significant spectral interactions between cytochrome *a* and cytochrome a_3 are absent. Thus, the redox state of Cu_A and cytochrome *a* could affect the electron-binding properties of cytochrome a_3. Similarly, the redox state of Cu_A and cytochrome *a* could affect the ligand-binding properties of cytochrome a_3.

The CO-binding properties of the fully reduced and mixed-valence CO complexes appear to differ (cf. Table I). This difference, however, is partly of statistical origin. In a model where both Cu_A (site 1) and cytochrome *a* (site 2) can rapidly exchange electrons with cytochrome a_3, the observed dissociation constant of a ligand which only binds to reduced cytochrome a_3 will be:

$$K_{d,app} = K_d(1 + K_{1e} + K_{2e}) \quad (3)$$

where $K_{d,app}$ is the observed dissociation constant of the mixed-valence enzyme complex; K_d the dissociation constant of cytochrome a_3 and CO; $K_{1e,2e}$ the electron affinities of site 1,2 with respect to cytochrome a_3.

The association rate constants are affected in the same way:

$$k_{on,app} = k_{on}\left(\frac{1}{1 + K_{1e} + K_{2e}}\right) \quad (4)$$

The dissociation rate should not be affected. Although experimentally a small effect was found (cf. Table I), it should be noted that these dissociation rates could not be determined accurately.

The model can explain at least in part the difference in K_d and in k_{on} between the mixed-valence and fully reduced oxidase. From the kinetic data in Table I it is possible to calculate a value of $K_{1e} + K_{2e}$ that is equal to or larger than 0.2. From the thermodynamic relationship between redox-potential difference and equilibrium constant, it is possible to calculate the difference in redox potentials of the electron-accepting site and cytochrome a_3. If two redox sites are present with an equal electron affinity, the potentials of these sites are at most 60 mV lower than that of cytochrome a_3; with one redox site the difference is 40 mV.

In redox titrations the midpoint potential of Cu_A has invariably been determined as 260 mV. However, conflicting results were reported for the midpoint potentials of cytochrome *a* and cytochrome a_3, varying from 220 to 380 mV [14,15,29,31]. In the rapid electron-transfer reaction after photodissociation of mixed-valence CO oxidase, about 0.07 e^- were found to be transferred from cytochrome a_3 to Cu_A, giving $K_{1e} = 0.075$. From this and from the midpoint potential of Cu_A (260 mV) a value for cytochrome a_3 of about 330 mV can be calculated. However, K_{2e} is then approx. 0.12 and cytochrome *a* and cytochrome a_3 differ by only 55 mV in midpoint potential. This is not in agreement with the observation that cytochrome *c* oxidase titrates optically as two distinct components with potentials of 250 and 370 mV [14,15,29,31]. This same discrepancy led Wikström et al. [14] to the proposal of the so-called neo-classical model, in which the midpoint potentials of cytochrome *a* and cytochrome a_3 are interdependent. Our CO-binding results support this model, although we find a smaller value for K_{2e} (0.12 vs. 1). It is interesting to note that Carithers and Palmer [30] found a value of 0.68.

The rapid electron-transfer reaction with a rate of 7000 s^{-1} observed at 820 nm and in the Soret region is due to electron transfer from cytochrome a_3 to Cu_A^{2+} after photolysis of cytochrome a_3^{2+}-CO and can be summarised by the equation:

$$Cu_A^{2+}\ a_3^{2+}\text{-CO} \underset{CO}{\overset{light}{\rightleftharpoons}} Cu_A^{2+}\ a_3^{2+} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Cu_A^{1+}\ a_3^{3+}$$

It should be noted that the measured rate constant of 7000 s^{-1} corresponds to the sum of the individual rate constants k_1 and k_{-1}. In the reaction of fully reduced cytochrome *c* oxidase with oxygen, Greenwood and Gibson [2,32] found that after oxidation of cytochrome a_3, the 820 nm band decreases with a limiting rate constant of $6 \cdot 10^3$ s^{-1}. This is very close to the value we find for this process.

In our experiments, this rapid reaction is the first electron-transfer reaction observed after photodissociation of cytochrome $a_3^{2+} \cdot CO$. Thus, there is an electron pathway between Cu_A and the cytochrome a_3-Cu_B pair. This is in agreement with the conclusion of Greenwood and Gibson [2] that Cu_A is the natural electron donor to the cytochrome

a_3-Cu_B pair. The same authors also demonstrated [2] that cytochrome *a* donates electrons to Cu_A with a rate of 700 s^{-1}. In our studies this reaction interfered with the CO recombination reaction in the mixed-valence enzyme. Optical studies at other wavelengths might give data on this electron-transfer reaction from cytochrome a_3 to cytochrome *a*. Electron-transfer reactions similar to those in this paper were observed before in flash photolysis experiments of cytochrome *c* oxidase in the presence of nitric oxide [33]. It was shown that the electron-transfer reaction between cytochrome *a* and cytochrome a_3 occurs even at temperatures below 50 K, but the reaction was found to be temperature dependent at these low temperatures. Thus, electron-transfer reactions in cytochrome *c* oxidase are coupled to a structural conformation of the enzyme.

Acknowledgements

The authors thank Mr. J.J. Schuurmans of the Vrije Universiteit (Amsterdam) for assistance in the early stage of this investigation, Mr. F.P.A. Mol, Mr. H.A. Prins and Mr. H. Gerritsen for their excellent technical assistance during the development of the laser-flash-photolysis equipment and Dr. H. Rademaker for considerable help with the curve-fitting programme. This study was in part supported by grants from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.) under the auspices of The Netherlands Foundation for Chemical Research (S.O.N.).

References

1 Van Gelder, B.F. and Muijsers, A.O. (1966) Biochim. Biophys. Acta 118, 47–57
2 Greenwood, C. and Gibson, Q.H. (1967) J. Biol. Chem. 242, 1782–1787
3 Chance, B., Leigh, J.S., Jr. and Waring, A. (1977) in Structure and Function of Energy-Transducing Membranes (Van Dam, K. and Van Gelder, B.F., eds), BBA Library 14, pp. 1–10, Elsevier, Amsterdam
4 Wever, R., Van Drooge, J.H., Muijsers, A.O., Bakker, E.P. and Van Gelder, B.F. (1977) Eur. J. Biochem. 73, 149–154
5 Wilson, D.F. and Miyata, Y. (1977) Biochim. Biophys. Acta 461, 218–230
6 Babcock, G.T., Vickery, L.E. and Palmer, G. (1978) J. Biol. Chem. 253, 2400–2411
7 Greenwood, C., Wilson, M.T. and Brunori, M. (1974) Biochem. J. 137, 205–215
8 Chance, B. (1953) J. Biol. Chem. 202, 407–416
9 Clore, G.M. and Chance, E.M. (1978) Biochem. J. 173, 799–810
10 Clore, G.M. and Chance, E.M. (1978) Biochem. J. 173, 811–820
11 Shaw, R.W., Hansen R.E. and Beinert, H. (1979) Biochim. Biophys. Acta 548, 386–396
12 Clore, G.M., Andréasson, L.-E., Karlsson, B., Aasa, R. and Malmström, B.G. (1980) Biochem. J. 185, 155–167
13 Boelens, R. and Wever, R. (1979) Biochim. Biophys. Acta 547, 296–310
14 Wikström, M.K.F., Harmon, H.J., Ingledew, W.J. and Chance, B. (1976) FEBS Lett. 65, 259–277
15 Erecinska, M. and Wilson, D.F. (1978) Arch. Biochim. Biophys. 188, 1–14
16 Wever, R. and Van Gelder, B.F. (1974) Biochim. Biophys. Acta 368, 311–317
17 Wever, R., Van Drooge, J.H., Van Ark, G. and Van Gelder, B.F. (1974) Biochim. Biophys. Acta 347, 215–223
18 Van Buuren, K.J.H. (1972) Binding of Cyanide to Cytochrome aa_3, Ph. D. Thesis, University of Amsterdam, Gerja, Waarland
19 Hartzell, C.R., Beinert, H., Van Gelder, B.F. and King, T.E. (1978) Methods Enzymol. 53, 54–66
20 Van Gelder, B.F. (1966) Biochim. Biophys. Acta 118, 36–46
21 Antonini, E. and Brunori, M. (1971) Hemoglobin and Myoglobin in Their Reactions with Ligands, North Holland, Amsterdam, pp. 167–169
22 Horecker, B.L. and Kornberg, A. (1948) J. Biol. Chem. 175, 385–390
23 Boelens, R. and Wever, R. (1980) FEBS Lett. 116, 223–226
24 Wilms, J., Dekker, H.L., Boelens, R. and Van Gelder, B.F. (1981) Biochim. Biophys. Acta 637, 168–176
25 Brunori, M., Bonaventura, J., Bonaventura, C., Antonini, E. and Wyman, J. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 868–871
26 Bonaventura, C., Bonaventura, J., Antonini, E., Brunori, M. and Wyman, J. (1973) Biochemistry 12, 3424–3428
27 Vanneste, W.H. (1966) Biochemistry 5, 838–848
28 Malmström, B.G. (1974) Q. Rev. Biophys. 6, 389–431
29 Babcock, G.T. Vickery, E.E. and Palmer, G. (1976) J. Biol. Chem. 251, 7907–7919
30 Carithers, R.P. and Palmer, G. (1981) J. Biol. Chem. 256, 7967–7976
31 Van Gelder, B.F., Van Rijn, J.L.M.L., Schilder, G.J.A. and Wilms, J. (1977) in Structure and Function of Energy-Transducing Membranes (Van Dam, K. and Van Gelder, B.F., eds.), BBA Library 14, pp. 61–68, Elsevier, Amsterdam
32 Gibson, Q.H. and Greenwood, C. (1965) J. Biol. Chem. 240, 2694–2698
33 Boelens, R., Rademaker, H., Pel, R. and Wever, R. (1982) Biochim. Biophys. Acta 679, 84–94