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EPR STUDIES OF THE PHOTODISSOCIATION REACTIONS OF CYTOCHROME *c* OXIDASE-NITRIC OXIDE COMPLEXES

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Three complexes of NO with cytochrome *c* oxidase are described which are all photodissociable at low temperatures as measured by EPR. The EPR parameters of the cytochrome a_3^{2+}-NO complex are the same both in the fully reduced enzyme and in the mixed-valence enzyme. The kinetics of photodissociation of cytochrome a_3^{2+}-NO and recombination of NO with cytochrome a_3^{2+} (in the 30–70 K region) revealed no differences in structure between cytochrome a_3^{2+} in the fully reduced and the mixed-valence states. The action spectrum of the photodissociation of cytochrome a_3^{2+}-NO as measured by EPR has maxima at 595, 560 and 430 nm, and corresponds to the absorbance spectrum of cytochrome a_3^{2+}-NO. Photodissociation of cytochrome a_3^{2+}-NO in the mixed-valence enzyme changes the EPR intensity at *g* 3.03, due to electron transfer from cytochrome a_3^{2+} to cytochrome a^{3+}. The extent of electron transfer was found to be temperature dependent. This suggests that a conformational change is coupled to this electron transfer. The complex of NO with oxidized cytochrome *c* oxidase shows a photodissociation reaction and recombination of NO (in the 20–40 K region) which differ completely from those observed in cytochrome a_3^{2+}-NO. The observed recombination occurs at a temperature 15 K lower than that found for the cytochrome a_3^{2+}-NO complex. The action spectrum of the oxidized complex shows a novel spectrum with maxima at 640 and below 400 nm; it is assigned to a Cu_B^{2+}-NO compound. The triplet species with $\Delta m_s = 2$ EPR signals at *g* 4 and $\Delta m_s = 1$ signals at *g* 2.69 and 1.67, that is observed in partially reduced cytochrome *c* oxidase treated with azide and NO, can also be photodissociated.

Introduction

Cytochrome *c* oxidase (ferrocytochrome *c* : oxygen oxidoreductase, EC 1.9.3.1) is a mitochondrial enzyme that catalyzes the electron transfer from cytochrome *c* to O_2. The enzyme contains four redox centres: a ligand-binding haem *a* group associated by definition with cytochrome a_3, a non-ligand binding haem *a* group associated with cytochrome *a*, and two non-equivalent copper atoms [1]. It is generally assumed that cytochrome *a* is the electron-accepting site in the reaction of the enzyme with reduced cytochrome *c* [2] and that cytochrome a_3 binds and reduces O_2 [3]. However, the function of the copper redox centres in the catalytic mechanism has not yet been definitely resolved. One of the copper atoms (Cu_A) is probably involved in the electron transfer from cytochrome *a* to cytochrome a_3 [4], while the other (Cu_B) is situated close to cytochrome a_3 [5,6] and has probably a function in the two-electron reduction of O_2 [7].

Electron transfer between the redox centres of cytochrome *c* oxidase was observed after binding CO and NO to cytochrome a_3 in the partially reduced enzyme [8,9] and reverse flow could be observed upon photodissociation of mixed-valence carboxy-cytochrome *c* oxidase in both the absence and presence of added ligands such as formate and azide [10–13]. In these experiments it could clearly be shown that also Cu_A was involved in the electron

transfer. The properties of Cu_B are less accessible to investigation. From EPR experiments [5] and magnetic susceptibility measurements [14], it followed that this copper atom is coupled anti-ferromagnetically to cytochrome a_3 in the oxidized enzyme; from binding studies of CO during reductive titrations it was concluded that both cytochrome a_3 and Cu_B must be reduced for binding of the ligand [8,15].

Recently, it was suggested from EPR experiments with NO that in the oxidized enzyme NO can bind to Cu_B^{2+}, breaking the anti-ferromagnetic coupling and revealing a high-spin haem signal assigned to cytochrome a_3^{3+} [16,17]. Through a one-electron reaction in the presence of NO and azide the cytochrome a_3-Cu_B complex can be converted into a state with a characteristic triplet EPR signal that is probably due to the formation of a cytochrome a_3^{2+}-NO($S = 1/2$)-Cu_B^{2+}($S = 1/2$) compound [16,18,19].

Addition of NO to reduced cytochrome *c* oxidase gives a cytochrome a_3^{2+}-NO complex that has a characterisitic EPR spectrum with nine hyperfine lines due to two non-equivalent nitrogen ligands of the haem. One of the nitrogen ligands is assigned to NO, the other to an axial imidazole group of a histidine in cytochrome a_3 [20–22].

Spectroscopic studies of this complex [23] have shown that cytochrome a_3^{2+}-NO can be photodissociated. Similar photodissociation experiments with cytochrome a_3^{2+}-CO showed that electron transfer occurs after photodissociation of the mixed-valence CO enzyme. Therefore, the EPR properties of various NO-cytochrome *c* oxidase complexes were studied before and after photodissociation.

In this paper it is shown that the Cu_B^{2+}-NO complex of the oxidized cytochrome *c* oxidase can be photodissociated also, but that this complex has completely different kinetic and optical properties as compared to the cytochrome a_3^{2+}-NO complex. Further experiments with mixed-valence NO-cytochrome *c* oxidase revealed that the extent of electron transfer between cytochrome *a* and cytochrome a_3 is temperature dependent.

Materials and Methods

Cytochrome *c* oxidase was prepared according to the method of Fowler et al. [24] as modified in our laboratory [25,26]. The absorption coefficient of cytochrome *c* oxidase (reduced minus oxidized) was 24.0 $mM^{-1} \cdot cm^{-1}$ at 605 nm [27]. Chemicals were of analar grade, mainly obtained from British Drug Houses. NADH, grade 2, was from Boehringer; its concentration was determined spectrophotometrically with $\epsilon = 6.22$ $mM^{-1} \cdot cm^{-1}$ at 340 nm [28]. Phenazine methosulphate was from Sigma. NO (Baker Chemicals) was purified by leading the gas through 1 M KOH or by freezing the higher oxides in a cold trap (−20°C). The solubility of NO in H_2O at 20°C is 2 mM at a pressure of 100 kPa [29].

All experiments were carried out anaerobically in Thunberg cuvettes or modified EPR tubes equipped with a special gas holder for anaerobic addition of NO. Anaerobiosis and reduction of the samples were carried out as described previously [12,13].

Optical spectra were obtained with a Cary 219 recording spectrophotometer. EPR spectra were obtained with a Varian E-9 spectrometer, coupled to an HP 2100 computer via a PDP 11-03 microcomputer. Magnetic field and microwave frequency were measured as described before [10]. The temperature of the EPR system was calibrated with an EPR tube in which two carbon resistors were present, one below and one above the centre of the cavity. During the experiments the temperature was measured with a carbon resistor placed directly underneath the EPR sample, as described in Ref. 30. The typical temperature gradient was 0.7 K/cm. Illumination was performed by irradiation through a light guide on the grid of the cavity, with filtered light of a 150 W xenon lamp or a 500 W slide projector. The light intensity was measured with a Photometer/Radiometer (model 450, E.G. and G.). Light intensities were varied by using a calibrated set of neutral density filters (Oriel). The wavelength of the irradiated light was varied by using different interference filters (Balzers). The temperature increase of the sample during illumination was checked by the Curie behaviour of the *g* 3.03 EPR signal in oxidized cytochrome *c* oxidase and found to be less than 2 K.

If not specified in the legends to the figures, cytochrome *c* oxidase was dissolved in 50 mM potassium phosphate (pH 7.4) and 0.5% Tween 80.

Results

When NO was added to reduced cytochrome *c* oxidase, we observed an EPR spectrum (Fig. 1A) of

the cytochrome a_3^{2+}-NO complex with nine characteristic hyperfine lines, which is in agreement with the result in Refs. 20–22. However, under our measuring conditions (temperature 15 K, modulation 10^{-3} T, microwave power 2 mW) the signals are strongly broadened. Illumination of this protein-NO complex at temperatures below 40 K resulted in an irreversible disappearance of the observed EPR spectrum (Fig. 1B). When the sample was heated to temperatures higher than 60 K, the EPR spectrum of cytochrome a_3^{2+}-NO reappeared (Fig. 1C). It is likely that these effects are due to photodissociation, since it has also been shown in optical experiments [23] that the cytochrome a_3^{2+}-NO complex can be photodissociated. When the experiment was done below 8 K, a broad signal around g 2 appeared concomitantly with the disappearance of the cytochrome a_3^{2+}-NO signal, as already described in Ref. 23 (data not shown). In contrast to the findings of these authors, however, the saturation behaviour of this new signal is not similar to that of Cu_A^{2+}: at 8 K it was hardly saturated at powers less than 1 mW, whereas at this temperature Cu_A^{2+} started saturating at 0.01 mW. At temperatures higher than 8 K the signal could not be observed.

In the temperature region between 30 and 55 K not all dissociated cytochrome a_3^{2+}-NO molecules recombined after photodissociation: only a fraction was reversibly dissociated. Fig. 2 shows the fraction of molecules that recombined after photodissociation in the EPR experiment, recorded at various temperatures. The temperature at which 50% of the molecules could recombine was 44 K, which is considerably lower than the value of 160 K at which the cytochrome a_3^{2+}-CO complex starts to recombine [31]. This transition temperature is also 20 K lower than that reported for the cytochrome a_3^{2+}-NO complex in optical experiments [23]. Since this difference could be caused by a difference in NO concentration in the two kinds of experiments, the recombination reaction of the cytochrome a_3^{2+}-NO complex was studied at various concentrations of NO and at various temperatures (Fig. 3). It was not observed, however, that the kinetics depended on the concentration of NO. The

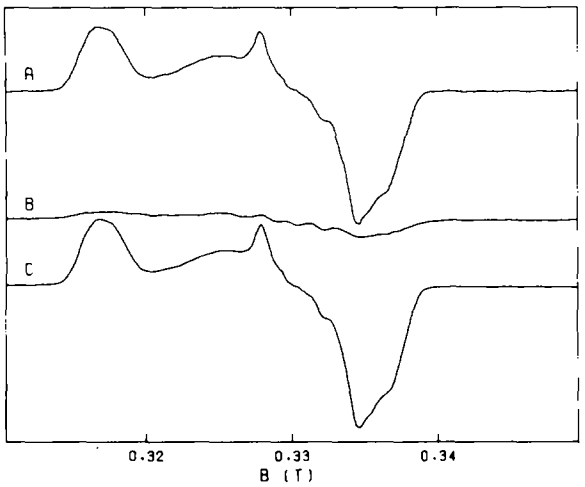

Fig. 1. The effect of light on the EPR spectrum of reduced cytochrome c oxidase in the presence of NO. (A) 0.35 mM cytochrome c oxidase reduced with NADH and phenazine methosulphate ($5e^-$/cytochrome aa_3) in 50 mM potassium phosphate (pH 7.4), 0.5% Tween 80, p_{NO} = 14 kPa. (B) After illumination of (A) at 18 K. (C) After warming (B) to 77 K. Conditions of EPR spectroscopy: frequency, 9.3326 GHz; microwave power, 2 mW; modulation amplitude, 10^{-3} T; scanning rate, 0.25 T/min; time constant, 0.3 s; temperature, 18 K.

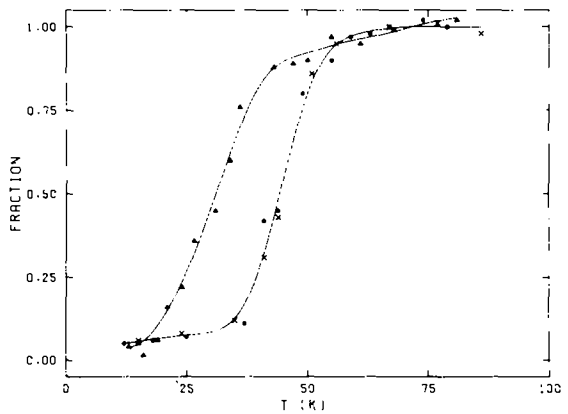

Fig. 2. Fraction of undissociated NO complexes of cytochrome c oxidase after photodissociation at various temperatures. The fraction was calculated by dividing the intensity of the EPR signal obtained 5 min after illumination, by the intensity before illumination. x, fully reduced ($5e^-$/cytochrome aa_3); •, mixed-valence ($2e^-$/cytochrome aa_3); ▲, oxidized cytochrome c oxidase. Conditions: 0.35 mM cytochrome c oxidase, 50 mM potassium phosphate (pH 7.4); 0.5% Tween 80, p_{NO} = 70 kPa, reduction by NADH and phenazine methosulphate. EPR conditions (except for the temperature) for the fully reduced and mixed-valence enzyme as in Fig. 1 and for the oxidized enzyme as in Fig. 7.

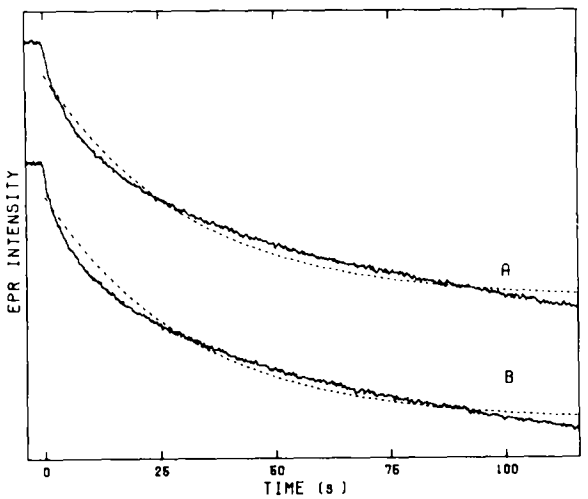

Fig. 3. Recombination reaction of NO with cytochrome a_3^{2+} in fully reduced cytochrome *c* oxidase after photodissociation at 50 K, observed with EPR at *g* 1.99. (A) p_{NO} = 0.7 kPa, (B) p_{NO} = 14 kPa. (------) Least-squares fit to an exponential decay. Conditions: as in Fig. 1. EPR conditions: frequency, 9.3332 GHz; microwave power, 2 mW; modulation amplitude, 10^{-3} T; field set, 0.3355 T; time constant, 0.3 s.

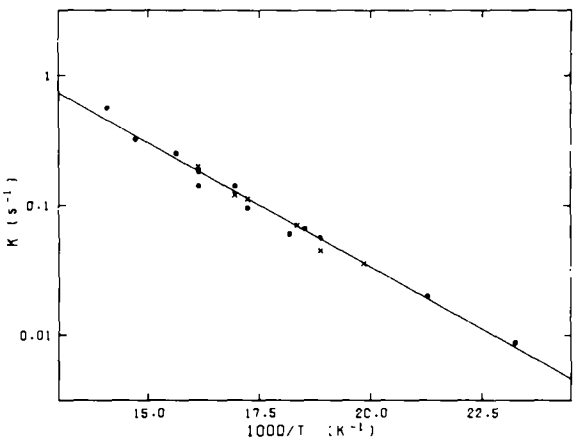

Fig. 4. Temperature dependence of the recombination rate of NO with cytochrome a_3^{2+} in fully reduced (•) and mixed-valence (x) cytochrome *c* oxidase. The rates were estimated from the half-times of the recombination reaction, as observed with EPR. EPR conditions as in Fig. 3.

observed recombination reaction was also not a simple exponential. Similar non-exponential kinetic behaviour has been observed for other haemoproteins, where at low temperatures also no concentration dependence is observed [32]. This suggests that the binding of NO to cytochrome a_3^{2+} is not very different from the ligand binding to other haemoproteins and that in cytochrome *c* oxidase also an internal pocket is present that can only contain one NO molecule. At these low temperatures the pocket is not accessible to NO molecules outside the pocket, which could explain the concentration independence.

The temperature dependence of the recombination rates, as estimated from the half-times of the observed kinetics, has been represented in an Arrhenius plot (Fig. 4). The activation energy is found to be 3.5 kJ/mol. The non-exponential recombination kinetics can be explained by inhomogeneity in this activation energy between the internal pocket and the Fe^{2+} of the haem.

Since it has been suggested in the literature [33,34] that the optical and kinetic properties of cytochrome a_3 change depending on the redox state of the other redox centres in cytochrome *c* oxidase, we also added NO to partially reduced ($2e^-$/cytochrome aa_3) cytochrome *c* oxidase. Upon addition of NO to partially reduced cytochrome *c* oxidase the signal around *g* 6, assigned to cytochrome a_3^{3+}, disappeared completely. Concomitantly, the EPR signal of cytochrome a^{3+} (*g* 3.03) increased from 25 to 85% of the intensity of one haem, while Cu_A^{2+} (*g* 2.09) increased from 65 to 90%. The cytochrome a_3^{2+}-NO complex was formed completely, since the signal at *g* 2.09 corresponded to that of one haem. On the basis of the integrations it can be concluded that the sample consists mainly of the mixed-valence NO complex. Similar results were observed upon addition of CO [8,11] and NO [9] to the partially reduced enzyme and are explained by electron transfer from the other redox centres to cytochrome a_3 because of the high affinity of CO and NO for the reduced state of cytochrome a_3. The EPR spectrum of cytochrome a_3^{2+}-NO in the mixed-valence state could be obtained by subtracting the EPR spectrum of Cu_A^{2+} in the oxidized enzyme from that obtained when NO was added to partially reduced cytochrome *c* oxidase. The spectrum of cytochrome a_3^{2+}-NO had the same *g* values (g_x 2.09, g_y 2.00, g_z 2.005) and hyperfine splitting constants (A_{1z} = 2.03

mT, $A_{2z} = 0.68$ mT) in the mixed-valence state as in the fully reduced state (not shown). This is in contrast to the results of Wilson et al. [35] who found differences in hyperfine structure in the z direction.

Photodissociation experiments of this mixed-valence NO-cytochrome c oxidase did not reveal a difference in kinetic behaviour. As shown in Fig. 2, the temperature at which NO recombined with 50% of cytochrome a_3^{2+} in the fully reduced and mixed-valence states did not differ. Furthermore, the estimated activation energy of the recombination reaction is the same for both species (Fig. 4).

The dissociation rates, k, of photolabile haem-ligand complexes are proportional to the light intensity I [36]: $k = \epsilon\phi I$, where ϵ is the absorbance coefficient of the molecule and ϕ the quantum yield (i.e., the ratio of the number of photodissociated molecules to the number of excited molecules). Fig. 5 shows the dependence of the dissociation rate on the light intensity ($\lambda = 590$ nm) for cytochrome a_3^{2+}-NO in the fully reduced and the mixed-valence states. Since the same slopes were observed within the error of the experiment, it may be concluded that there are no great differences in optical properties assuming that the quantum yield is equal in both enzyme species. Because the quantum yield of the photodissociation reaction of a haem-ligand complex is nearly constant in the visible region [37], the action spectrum of cytochrome a_3^{2+}-NO can be obtained by irradiation with light of various wavelengths and measurement of the dissociation rate. Fig. 6 shows that the action spectra of cytochrome a_3^{2+}-NO as measured by EPR have absorption maxima at 430, 560 and 595 nm, corresponding to those in the optical spectrum of cytochrome a_3^{2+}-NO. It is obvious that the spectra are the same for cytochrome a_3^{2+}-NO in the fully reduced and the mixed-valence states. The spectra are very similar to the photochemical action spectrum of cytochrome a_3^{2+}-CO [38].

Although no differences in kinetic and optical properties of cytochrome a_3^{2+}-NO in the fully reduced and the mixed-valence states were observed, there is a difference in effect of illumination of the mixed-

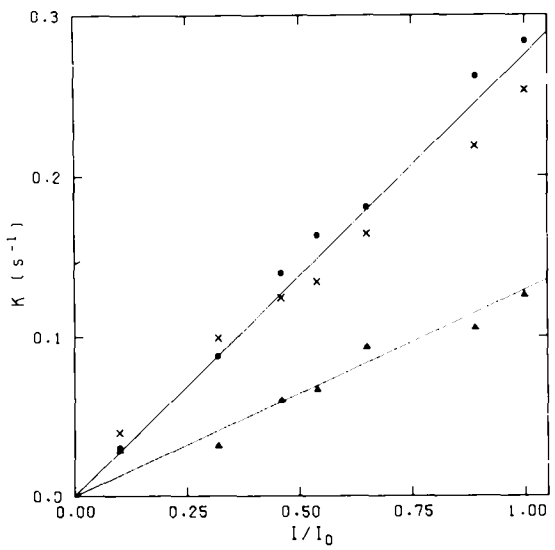

Fig. 5. Dependence of the dissociation rates on the light intensities during illumination of NO-cytochrome c oxidase complexes. The dissociation rates were calculated from the half-times of the dissociation reaction, x, fully reduced; •, mixed-valence; and ▲, fully oxidized cytochrome c oxidase. Conditions: as in Fig. 2. The irradiation wavelength was 590 nm. The ligh-induced dissociation in the fully reduced and mixed-valence enzyme was followed at g 2.117 (B = 0.3150 T) and in the oxidized enzyme at g 6.196 (B = 0.1076 T).

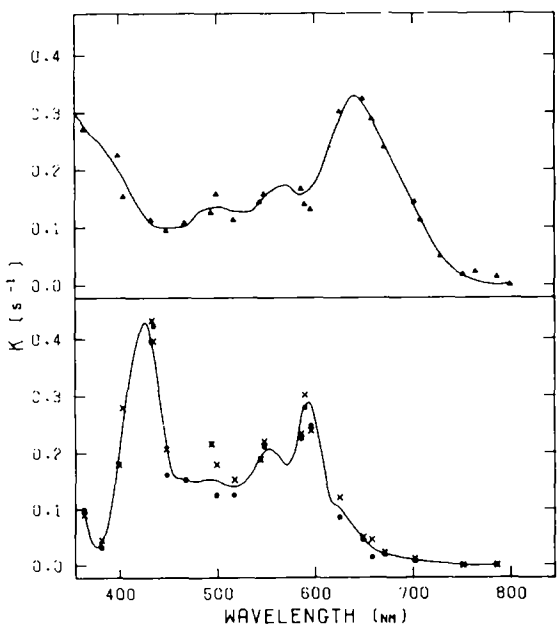

Fig. 6. Action spectra of the photodissociation reactions in cytochrome c oxidase-NO complexes. x, fully reduced; •, mixed-valence; and ▲, fully oxidized cytochrome c oxidase. Conditions: as in Fig. 5.

valence NO enzyme as compared to the fully reduced enzyme. The intensity of the EPR signal assigned to cytochrome a^{3+} (g 3.03) changes depending on the temperature at which the illumination was carried out. After illumination of the mixed-valence NO enzyme at 20 K a 5% decrease in g 3.03 intensity was observed. However, when the sample was warmed to 40 K and illuminated, the signal decreased by about 10%; when the sample was illuminated at 50 K and slowly cooled to 20 K under illumination, a 15% decrease in intensity of the g 3.03 peak was observed. No changes occurred under these conditions in the Cu_A^{2+} signal (g 2.17). When the sample previously illuminated at 50 K was allowed to warm up to 60 K and cooled in the dark to 20 K, the cytochrome a^{3+} intensity was restored completely. Illumination of the mixed-valence NO enzyme at 8 K resulted in an increase in the broad signal at g 2 (similar to the reduced enzyme), but under these conditions the signal at g 3.03 was not affected. Since under the measuring conditions before and after illumination the g 3.03 signal was not saturated, it is concluded that cytochrome a^{3+} is reduced after photodissociation of cytochrome a_3^{2+}-NO in the mixed-valence enzyme. However, no new EPR signals (from either cytochrome a_3^{3+} or Cu_B^{2+}) were observed and the source of this light-induced reduction of cytochrome a is therefore still unclear.

Two other cytochrome c oxidase-NO complexes have been described in the literature [16,17,19]. When NO is added to oxidized cytochrome c oxidase in the absence or presence of excess ferricyanide, a characteristic, almost axial haem signal is formed in addition to the signals assigned to cytochrome a^{3+} and Cu_A^{2+}. This signal can only be assigned to cytochrome a_3 in a high-spin state and corresponded to 15% of the intensity of one haem (Fig. 7A). When this cytochrome c oxidase-NO complex was illuminated below 20 K, the axial g 6 signal disappeared irreversibly (Fig. 7B). Warming the sample to 40 K restored the cytochrome a_3^{3+} signal (Fig. 7C). The temperature at which 50% of the EPR signal reappeared (31 K) is very different from that of the cytochrome a_3^{2+}-NO complex (Fig. 2). The rates of formation of the g 6 signal showed almost no temperature dependence, the activation energy being less than 0.3 kJ/mol. Fig. 5 shows that the dissociation rates for the complex are linearly dependent on

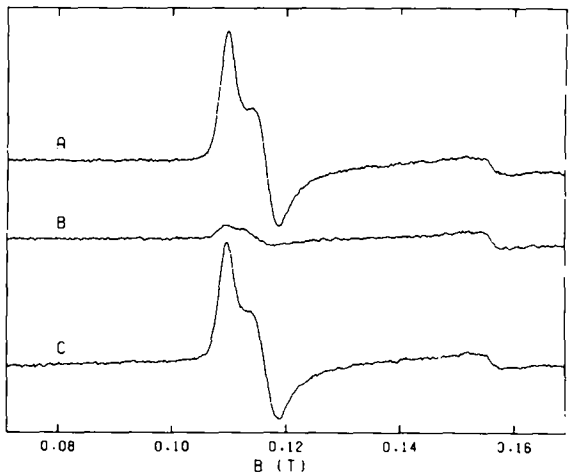

Fig. 7. The effect of light on the EPR spectrum of oxidized cytochrome c oxidase in the presence of NO. (A) 0.35 mM cytochrome c oxidase in 50 mM potassium phosphate (pH 7.4), 0.5% Tween 80, p_{NO} = 70 kPa. (B) After illumination of (A) at 15 K. (C) After warming of (B) to 40 K. Conditions of EPR spectroscopy: frequency, 9.332 GHz; microwave power, 2 mW; modulation amplitude, 10^{-3} T; scanning rate, 0.5 T/min; time constant, 0.3 s; temperature, 15 K.

light intensity (λ = 590 nm), as also found for cytochrome a_3^{2+}-NO. However, since the slopes of the lines are different, the light-absorbing complex must have optical properties different from those of cytochrome a_3^{2+}-NO. But it is also possible that the quantum yield of both complexes differs. The dissociation rates of this cytochrome (a_3^{3+}-Cu_B^{2+})-NO complex were, therefore, measured at a number of wavelengths (Fig. 6). The observed action spectrum of this complex, with maxima at 640 and below 400 nm, does not resemble the absorbance spectrum of a haem compound and is completely different from that of cytochrome a_3^{2+}-NO. The spectrum has the properties of a Cu^{2+} spectrum [39]. Complexes of NO with copper-proteins show a weak band at 610 nm [40,41] and model complexes of NO with copper show also a charge-transfer band at about 600 nm [42], the intensity of which depends on other ligands. Therefore, we assigned the observed action spectrum to Cu_B^{2+}-NO.

Since the EPR photodissociation experiments indicated the existence of a complex of NO with Cu_B^{2+}, as already suggested in Ref. 16, we also studied the optical spectrum of the oxidized enzyme-NO

complex. Upon addition of NO to oxidized cytochrome *c* oxidase a series of optically detectable reactions occur. Immediately after addition of NO to oxidized cytochrome *c* oxidase in the presence of excess ferricyanide an absorbance band was observed at 605 nm (Fig. 8, dotted line). Since this absorbance increase runs parallel to a 3.5-fold larger absorbance increase at 445 nm and to a decrease in the *g* 3.03 EPR signal, the spectral changes have been assigned to partial reduction of cytochrome *a*. This form of the enzyme is not stable in the presence of ferricyanide. After the oxidation of cytochrome *a* by ferricyanide ($k = 2.1 \cdot 10^5\ M^{-1} \cdot s^{-1}$), a relatively stable complex is formed which has no large absorbance differences as compared to the oxidized enzyme (Fig. 8, dashed line). Under these conditions (cf. Fig. 6), the Cu_B^{2+}-NO complex is present.

In the presence of ferricyanide this complex was slowly reduced ($t_{1/2} = 2000$ s), forming a cytochrome a_3^{2+}-NO complex with absorbance maxima at 595, 560 and 430 nm (Fig. 8, unbroken line). In the EPR spectrum the cytochrome a_3^{2+}-NO complex also became visible. In the absence of ferricyanide, however, the change at 605 nm was not clearly separated from the change at 595 nm, since the formation of cytochrome a_3^{2+}-NO was strongly accelerated.

When azide is added to the oxidized cytochrome *c* oxidase-NO complex, a triplet EPR spectrum is formed [16,19], with a half-field signal at *g* 4 showing copper hyperfine splitting of $98 \cdot 10^{-4}$ T. Also this complex is photodissociable. Fig. 9 shows the difference between the EPR spectrum recorded before and after illumination, indicating disappearance of this triplet species. When the sample was warmed to 40 K, the triplet species was not formed again. Warming to 77 K revealed the appearance of a cytochrome a_3^{3+}-N_3^- signal at *g* 2.75 [11]. However, after thawing and freezing, the cytochrome a_3^{3+}-N_3^- signal disappeared and the triplet signal was formed again.

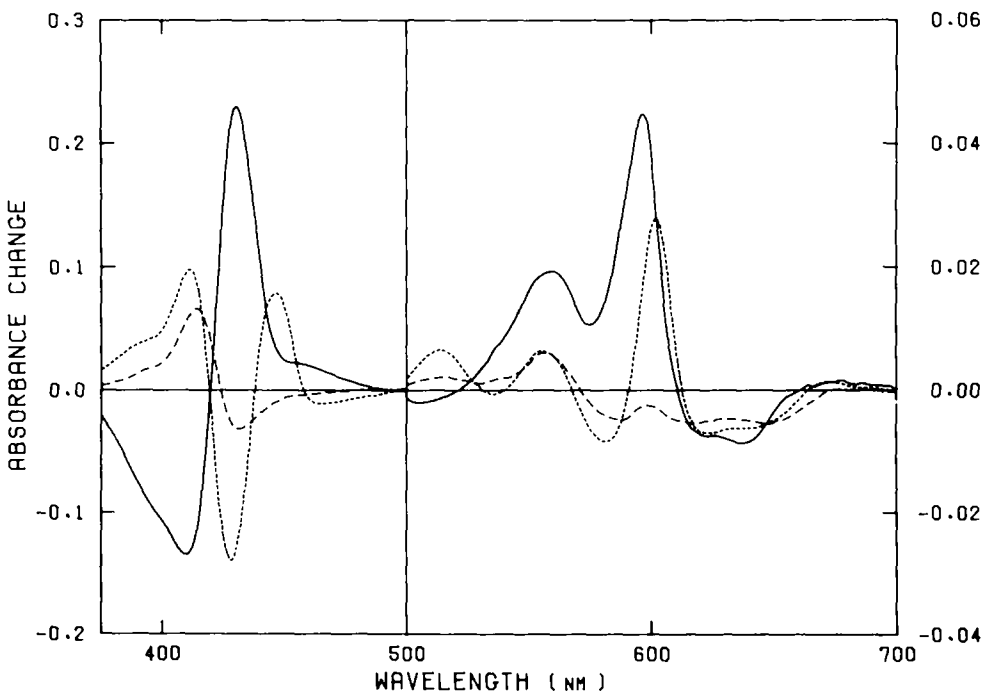

Fig. 8. The effect of NO on the absorbance spectra of oxidized cytochrome *c* oxidase. The spectra were recorded as difference spectra with oxidized cytochrome *c* oxidase as a reference. Conditions: 10 μM cytochrome *c* oxidase, 100 mM potassium phosphate (pH 7.4), 1% Tween 80; $p_{NO} = 4$ kPa. (- - - - - -) Directly after addition of NO; (– – –) in the presence of 160 μM ferricyanide after 2 min; (———) after 10 h.

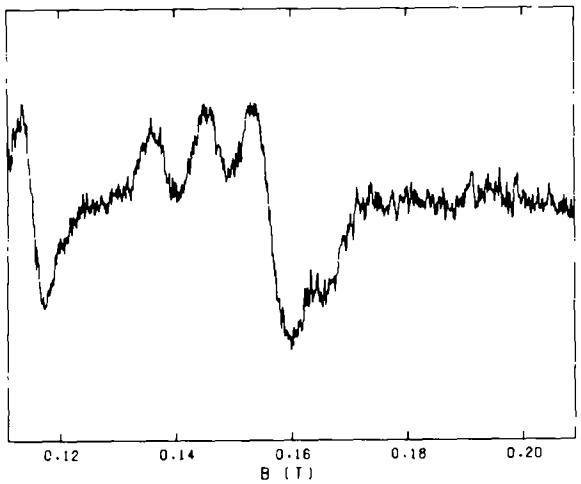

Fig. 9. The effect of illumination on the half-field region of the EPR spectrum of cytochrome *c* oxidase in the presence of NO and azide. The spectrum shown is the difference between the EPR spectrum recorded before illumination and the spectrum after illumination. Conditions: 0.35 mM cytochrome *c* oxidase in 50 mM potassium phosphate (pH 7.4), 0.5% Tween 80, incubated with (1) NO (p_{NO} = 50 kPa) and (2) NaN_3 (4.0 mM). EPR conditions: frequency, 9.24 GHz; microwave power, 33 mW; modulation amplitude, 10^{-3} T; scanning rate, 0.25 T/min; time constant, 0.3 s; temperature, 15 K.

The triplet signal can also be formed by addition of NO to a one-electron ($1e^-$/cytochrome aa_3)-reduced cytochrome *c* oxidase (data not shown), indicating that this signal is a result of a partially reduced cytochrome a_3-Cu_B-NO complex. The intensity of the signal obtained by this method was lower by about a factor 10 than that induced in the reaction of the enzyme with azide and NO.

Discussion

As is shown, three different NO-cytochrome *c* oxidase complexes exist which can all be dissociated by light. Fully reduced and half-reduced cytochrome *c* oxidase bind NO to form a paramagnetic cytochrome a_3^{2+}-NO complex ($S = 1/2$) which shows a characteristic EPR spectrum with nine hyperfine lines in the *z* direction: the larger splitting into three lines caused by the nuclear spin of the nitrogen of NO, and the subsequent smaller splitting into three lines due to another nitrogen that has been assigned to an axial imidazole group of a histidine [20–22]. The redox state of cytochrome *a* and Cu_A in cytochrome *c* oxidase had no influence on this EPR spectrum, indicating that the immediate environment of haem *a* in cytochrome a_3 does not change with the redox state of these redox centres, which is in contrast to what has been suggested in Ref. 35. This may also be concluded from the photodissociation behaviour and recombination kinetics of partially and fully reduced cytochrome *c* oxidase. These enzyme species show the same action spectra on photodissociation, which also means that the energy taken up by cytochrome *a* and Cu_A during illumination is not transmitted to cytochrome a_3^{2+}-NO at low temperatures. The observed recombination kinetics with NO at about 50 K do not reveal differences in the direct environment of haem *a* in cytochrome a_3. The rates are independent of the NO concentration, indicating that only one NO molecule is involved in the binding process, both in the fully reduced and in the mixed-valence states.

The observed non-exponential behaviour of the recombination process is a general property of the reaction of haemoproteins with ligands at low temperatures and can be explained by a spectrum of activation energies [32,43]. The average activation energy of the NO recombination reaction is higher than that for the NO recombination with haemoglobin and myoglobin, as is also reflected from the high recombination temperature of cytochrome a_3^{2+}-NO as compared to other proteins [44]. The same phenomenon was observed in the recombination reaction of cytochrome a_3 with CO ($T_{1/2}$ = 180 K) as compared with myoglobin and haemoglobin ($T_{1/2}$ = 25–30 K). Yonetani et al. [45] explained this by assuming that after photodissociation part of the protein was inserted as a barrier between CO and cytochrome a_3. The same was observed in the present experiments for the recombination of cytochrome a_3 with NO, though with a very different activation energy (3.5 kJ/mol as compared to 36 kJ/mol) and at temperatures where there is much less thermal mobility ($T_{1/2}$ = 44 K vs. 180 K). It is more likely, therefore, that the special behaviour of cytochrome *c* oxidase in photodissociation reactions is directly due to the properties of the ligand-binding site: close proximity of haem *a* and Cu_B. After photodissociation of CO or NO from the haem iron of cyto-

chrome a_3, the ligand can still be bound by Cu_B as suggested in Refs. 23 and 44. In such a model, where the ligands bridge between the haem *a* of cytochrome a_3 and Cu_B [16], photodissociation would only lead to a small displacement or a rotation of the ligand. The difference in recombination kinetics of CO and NO must then be explained by a difference in relative affinities for both redox centres.

An EPR-detectable complex is formed after photodissociation of cytochrome a_3^{2+}-NO in both the fully reduced and the mixed-valence enzyme. This signal has been assigned to the formation of a Cu_B^{2+}-NO^- complex [23]. The signal is only visible at temperatures below 15 K and does not show copper hyperfine structure such as the reaction intermediate observed when reduced cytochrome *c* oxidase reacts with O_2 [46], nor does it show an EPR spectrum similar to the Cu^{2+}-NO^- species of ceruloplasmin [47].

The photodissociation of half-reduced cytochrome *c* oxidase-NO further revealed that electron transfer occurs from cytochrome a_3 to cytochrome *a* after illumination. In contrast to similar experiments with CO [48] no EPR signals of cytochrome a_3^{3+} became visible. This might be explained by a magnetic coupling between NO (eventually in a Cu_B^{2+}-NO^- complex) and cytochrome a_3^{3+}, resulting in a diamagnetic ($S = 0$) or EPR-invisible ($S = 2$) complex. The temperature dependence of the intensity decrease at *g* 3.03 after photodissociation shows that the electron transfer is coupled to a conformational change of the enzyme and is not a simple type of electron tunnelling [49]. The reason why no parallel reduction of Cu_A^{2+} was observed is unclear, since Cu_A is normally involved in the electron transfer from cytochrome *a* to cytochrome a_3. An explanation may be that the temperature dependences of the midpoint potentials of various redox centres are very different from each other [50], resulting in hardly any reduction of Cu_A compared to cytochrome *a* at low temperatures. Another explanation may be that a drastic conformational change is associated with the reduction of Cu_A [51] which does not occur at low temperatures.

A fourth cytochrome *c* oxidase-NO complex is formed (which shows half-field EPR signals) when oxidized cytochrome *c* oxidase reacts with NO and azide [16,19] or when NO is added to one-electron-reduced cytochrome *c* oxidase. Similar half-field signals are observed upon addition of NO to ceruloplasmin [47] or haemocyanin [52]. This complex has the EPR properties of a triplet state ($S = 1$). In our photodissociation experiment the half-field signal ($\Delta m_s = 2$) with a hyperfine structure from one copper ($A = 98 \cdot 10^{-4}$ T) disappeared. Since the photolability of this species was not reversible at 31 K, as was observed with the Cu_B^{2+}-NO complex of the oxidized cytochrome *c* oxidase, nor at 44 K, at which the recombination of NO with cytochrome a_3^{2+} occurs, the definitive assignment of the binding site of NO within the cytochrome a_3-Cu_B pair remains difficult. At higher temperatures (77 K) the cytochrome a_3^{2+}-Cu_B^{2+} complex, formed after photodissociation of the triplet species, reacted with azide to produce an EPR-detectable stable cytochrome a_3^{3+}-N_3^--Cu_B^{1+} complex. Under these conditions the NO binding was inhibited and did not occur until the sample was thawed.

The present optical experiments show that under conditions when the oxidized cytochrome *c* oxidase-NO complex is formed, no large absorbance changes occur in the optical spectrum between 500 and 700 nm and that the Soret peak only shows a minor spectral shift to a lower wavelength. This complex has the characteristic high-spin EPR signal at *g* 6 that is the result of the breaking of anti-ferromagnetic coupling between cytochrome a_3^{3+} ($S = 1/2$) and Cu_B^{2+} ($S = 1/2$) because of the formation of a diamagnetic Cu_B^{2+}-NO complex [16]. From the action spectrum of the photodissociation rates of the oxidized cytochrome *c* oxidase-NO compound it can be concluded that a Cu_B-ligand complex is indeed formed, since this spectrum is very similar to those of other copper-protein spectra [39–41]. The maximum of the absorbance band lies between 620 and 650 nm and no strong absorbance changes can be detected optically under the conditions when this compound is formed.

The fact that Cu_B in cytochrome *c* oxidase can bind ligands gives interesting possibilities for the mechanism of the O_2 reaction, since it is known that almost all O_2-reducing oxidases are copper-proteins. This Cu_B might be involved in binding O_2^- after the first reduction step, as has been suggested from kinetic experiments [3,7]. Furthermore, the fact that the high-spin ($S = 5/2$) haem EPR spectrum disap-

pears when the copper-NO chromophore is illuminated at 10–25 K shows that cytochrome a_3 and Cu_B must be located very close to each other. This has already been suggested from EPR measurements and magnetic susceptibility measurements, where an antiferromagnetic interaction between cytochrome a_3 and Cu_B could be shown [5,6,14].

The simplest model of the O_2-binding site in which the haem iron of cytochrome a_3 as well as Cu_B can bind ligands is one in which the ligand, L (NO, CO or O_2), bridges between both metal ions: Fe^{2+} (cytochrome a_3)-L-Cu_B^+. A similar model can be proposed for the binding of NO to oxidized cytochrome *c* oxidase: Fe^{3+} (cytochrome a_3)-NO-Cu_B^{2+}. However, direct photodissociation of the Cu_B^{2+}-NO bond or photodissociation coupled with a charge transfer should then give an EPR signal of Cu_B^{2+} ($S = 1/2$) or cytochrome a_3^{3+}-NO^+ ($S = 5/2$), respectively. Since no extra EPR signals are observed after illumination, it is more probable that the coupling ligand which is responsible for the transmittance of the anti-ferromagnetic interaction between cytochrome a_3 and Cu_B in the oxidized enzyme is not replaced by NO upon addition of NO to the oxidized enzyme. NO will then be a non-bridging ligand bound to Cu_B^{2+} and after breaking of the Cu_B^{2+}-NO bond the original cytochrome a_3^{2+}-Cu_B^{2+} complex is re-formed. The coupling ligand responsible for the interaction could be a protein residue bound to cytochrome a_3 or some O_2 intermediate. Another possibility is that NO is a bridging ligand and that upon photodissociation of NO the coupling ligand returns to its original position. However, it is not very likely that at 30 K enough thermal energy is available to replace the coupling ligand. Upon reduction of cytochrome *c* oxidase the ligand bond to cytochrome a_3 is broken (possibly triggered by reduction of Cu_B), making cytochrome a_3 more accessible to ligand reactions. Such a mechanism might explain the difference in reactivity of the oxidized and partially reduced cytochrome *c* oxidase towards CN^- [53], and also that two electrons are necessary for the formation of a mixed-valence carboxy enzyme [8]. The observation of a Cu_B^{2+}-NO complex associated with cytochrome a_3 gives strong support to the recent ideas in which both cytochrome a_3 and Cu_B are involved in the O_2 reaction [7,46,54].

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References

1 Van Gelder, B.F. and Muijsers, A.O. (1966) Biochim. Biophys. Acta 118, 47–57
2 Gibson, Q.H., Greenwood, C., Wharton, D.C. and Palmer, G. (1965) J. Biol. Chem. 240, 888–894
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 Greenwood, C. and Gibson, Q.H. (1967) J. Biol. Chem. 242, 1782–1787
5 Van Gelder, B.F. and Beinert, H. (1969) Biochim. Biophys. Acta 189, 1–24
6 Palmer, G., Babcock, G.T. and Vickery, L.E. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 2206–2210
7 Denis, M. (1981) Biochim. Biophys. Acta 634, 30–40
8 Wever, R., van Drooge, J.H., Muijsers, A.O., Bakker, E.P. and Van Gelder, B.F. (1977) Eur. J. Biochem. 73, 149–154
9 Wever, R., Van Ark, G. and Van Gelder, B.F. (1977) FEBS Lett. 84, 388–390
10 Wever, R., Van Drooge, J.H., Van Ark, G. and Van Gelder, B.F. (1974) Biochim. Biophys. Acta 347, 215–223
11 Wever, R. and Van Gelder, B.F. (1974) Biochim. Biophys. Acta 368, 311–317
12 Boelens, R. and Wever, R. (1979) Biochim. Biophys. Acta 547, 296–310
13 Boelens, R. and Wever, R. (1980) FEBS Lett. 116, 223–226
14 Tweedle, M.F., Wilson, L.J., García-Iñiguez, L., Babcock, G.T. and Palmer, G. (1978) J. Biol. Chem. 253, 8065–8071
15 Lindsay, J.G., Owen, C.S. and Wilson, D.F. (1975) Arch. Biochem. Biophys. 169, 492–505
16 Stevens, T.H., Brudvig, G.W., Bocian, D.F. and Chan, S.I. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 3320–3324
17 Brudvig, G.W., Stevens, T.H., Morse, R.H. and Chan, S.I. (1981) Biochemistry 20, 3912–3921

18 Stevens, T.H., Brudvig, G.W. and Chan, S.I. (1980) Fed. Proc. 39, p. 2060, Abstr. 2399
19 Brudvig, G.W., Stevens, T.H. and Chan, S.I. (1980) Biochemistry 19, 5275–5285
20 Blokzijl-Homan, M.F.J. and Van Gelder, B.F. (1971) Biochim. Biophys. Acta 234, 493–498
21 Stevens, T.H., Bocian, D.F. and Chan, S.I. (1979) FEBS Lett. 97, 314–316
22 Stevens, T.H. and Chan, S.I. (1981) J. Biol. Chem. 256, 1069–1071
23 Yoshida, S., Hori, H. and Orii, Y. (1980) J. Biochem. 88, 1623–1627
24 Fowler, L.R., Richardson, S.H. and Hatefi, Y. (1962) Biochim. Biophys. Acta 64, 170–173
25 Van Buuren, K.J.H. (1972) Binding of Cyanide to Cytochrome aa_3, Ph. D. Thesis, University of Amsterdam, Gerja, Waarland
26 Hartzell, C.R., Beinert, H., Van Gelder, B.F. and King, T.E. (1978) Methods Enzymol. 53, 54–66
27 Van Gelder, B.F. (1966) Biochim. Biophys. Acta 118, 36–46
28 Horecker, B.L. and Kornberg, A. (1948) J. Biol. Chem. 175, 385–390
29 Antonini, E. and Brunori, M. (1971) Hemoglobin and Myoglobin in their Reactions with Ligands, North-Holland, Amsterdam
30 Albracht, S.P.J., Dooijwaard, G., Leeuwerik, F.J. and Van Swol, B. (1977) Biochim. Biophys. Acta 459, 300–317
31 Chance, B., Schoener, B. and Yonetani, T. (1965) in Oxidases and Related Redox Systems (King, T.E., Mason, H.S. and Morrison, M., eds.), pp. 609–621, Wiley, New York
32 Austin, R.H., Beeson, K.W., Eisenstein, L., Frauenfelder, H. and Gunsalus, I.C. (1975) Biochemistry 14, 5355–5373
33 Malmström, B.G. (1974) Q. Rev. Biophys. 6, 389–431
34 Greenwood, C., Wilson, M.T. and Brunori, M. (1974) Biochem. J. 137, 205–215
35 Wilson, D.F., Erecinska, M. and Owen, C.S. (1976) Arch. Biochem. Biophys. 175, 160–172
36 Noble, R.W., Brunori, M., Wyman, J. and Antonini, E. (1967) Biochemistry 6, 1216–1222
37 Setlow, R.B. and Pollard, E.C. (1962) Molecular Biophysics, pp. 306–347, Addison-Wesley, Reading, MA
38 Castor, L.N. and Chance, B. (1955) J. Biol. Chem. 217, 453–465
39 Brill, A.S. (1977) Transition Metals in Biochemistry, Springer-Verlag, Heidelberg
40 Van Leeuwen, F.X.R., Wever, R., Van Gelder, B.F., Avigliano, L. and Mondovi, B. (1975) Biochim. Biophys. Acta 403, 285–291
41 Van Leeuwen, F.X.R. and Van Gelder, B.F. (1978) Eur. J. Biochem. 87, 305–312
42 Williams, R.J.P. (1966) in The Biochemistry of Copper (Peisach, J., Aisen, P. and Blumberg, W.E., eds.), pp. 131–148, Academic Press, New York
43 Alberding, N., Chan, S.S., Eisenstein, L., Frauenfelder, H., Good, D., Gunsalus, I.C., Nordlund, T.M., Perutz, M.F., Reynolds, A.H. and Sorensen, L.B. (1978) Biochemistry 17, 43–51
44 Orii, Y. (1978) Adv. Biophys. 11, 285–308
45 Yonetani, T., Iizuka, T., Yamamoto, H. and Chance, B. (1973) in Oxidases and Related Redox Systems (King, T.E., Mason, H.S. and Morrison, M., eds.), pp. 401–405, University Park Press, Baltimore
46 Reinhammar, B., Malkin, R., Jensen, P., Karlsson, B., Andréasson, L.-E., Aasa, R., Vänngård, T. and Malmström, B.G. (1980) J. Biol. Chem. 255, 5000–5003
47 Van Leeuwen, F.X.R., Wever, R. and Van Gelder, B.F. (1973) Biochim. Biophys. Acta 315, 200–203
48 Leigh, J.S., Jr., Wilson, D.F., Owen, C.S. and King, T.E. (1974) Arch. Biochem. Biophys. 160, 476–486
49 Chance, B., De Vault, D.C., Frauenfelder, H., Marcus, R.A., Schrieffer, J.R. and Sutin, N. (1979) Tunneling in Biological Systems, Academic Press, New York
50 Larroque, C., Maurel, P. and Douzou, P. (1978) Biochim. Biophys. Acta 501, 20–32
51 Beinert, H. (1980) Coord. Chem. Rev. 33, 55–85
52 Schoot Uiterkamp, A.J.M., Van der Deen, H., Berendsen, H.C.J. and Boas, J.F. (1974) Biochim. Biophys. Acta 372, 407–425
53 Van Buuren, K.J.H., Zuurendonk, P.F., Van Gelder, B.F. and Muijsers, A.O. (1972) Biochim. Biophys. Acta 256, 243–257
54 Nicholls, P. and Chanady, G.A. (1981) Biochim. Biophys. Acta 634, 256–265