Biochimica et Biophysica Acta, 591 (1980) 458—470

BBA 47868

STUDIES ON THE ORIGIN OF THE NEAR-INFRARED (800—900 nm) ABSORPTION OF CYTOCHROME *c* OXIDASE

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(Received November 23rd, 1979)

Key words: Cytochrome c oxidase; ESR; Cu oxidation state; Mixed valence state; (Low temperature reflectance spectroscopy)

Summary

Data are presented which were collected in the course of the past ten years and bear on the correlation of absorbance at 800 nm and the EPR signal at $g = 2$ ('copper signal') of cytochrome *c* oxidase in various states of oxidation and ligation. Both EPR and optical reflectance spectra were obtained at low temperature (—170 to —190°C). For some sets of samples spectra were recorded in the range 500—1100 nm. A particular effort was made to study this correlation with what are called 'mixed valence' states (Greenwood, C., Wilson, M.T. and Brunori, M. (1974) Biochem. J. 137, 205—215), when cytochrome *a* and the EPR-detectable copper are thought to be oxidized and the other components reduced and vice versa. These data show no evidence that the copper component of cytochrome oxidase which has so far not been detected by EPR makes a contribution to the absorption between 800 and 900 nm exceeding 10—15% of the total, which is close to or within the error of the respective measurements. For the various states of the oxidase examined in this work the 700—800 nm region did not appear to be more useful than the 800—900 nm region for determining the state of the EPR-undetectable copper in a reliable way. These conclusions are in agreement with results presented previously

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Abbreviation: Tricine, *N*-tris(hydroxymethyl)methylglycine.

The metal components of cytochrome oxidase are designated as follows: the cytochromes as *a* and a_3; EPR detectable and undetectable copper as Cu_d and Cu_u (also designated as Cu_{a_3} and Cu_{a_3} [14] or Cu_A and Cu_B by other authors). The mixed valence states ($a^{3+}Cu_d^{2+}a_3^{2+}Cu_u^{+}$ and $a^{2+}Cu_d^{+}a_3^{3+}Cu_u^{2+}$) will be designated as CO- and formate-mixed valence states, respectively, or CO and formate will follow 'mixed valence' in parenthesis.

from other laboratories concerning the relationship of optical (approx. 800 nm) and EPR spectroscopic ($g = 2$) data obtained with the enzyme.

Introduction

It is now generally accepted that there are two heme and two copper components per minimal functional unit of cytochrome *c* oxidase (EC 1.9.3.1). Neither heme nor copper components show identical spectroscopic or functional features. The investigation of the copper components has met with particular difficulties since the optical absorption spectra are dominated by the heme constituents and EPR spectroscopy has so far only shown one species of endogenous copper, qualitatively and quantitatively. The so called 'copper (EPR) signal' of cytochrome oxidase has very unusual properties and indeed it has been proposed that it does not, in fact, represent an EPR signal of the Cu^{2+} [1,2] in a strict sense, although it is agreed that in the absence of copper the signal would not be observed. In 1961, Griffiths and Wharton [3] drew attention to a relatively weak absorption band (approx. 2 $mM^{-1} \cdot cm^{-1}$) of cytochrome oxidase in the near infrared (800—900 nm). Wharton and Tzagoloff [4] adduced evidence that this absorption was due in large part to cupric copper in the enzyme. Following this, experiments in a number of laboratories including our own, confirmed this observation [4—10] and found good correlation of the intensity of this band with the intensity of the EPR signal attributed to copper, so that it was generally accepted that at least a sizeable fraction of this near-infrared absorption and the EPR signal were due to the same component of the enzyme. However, it has been pointed out repeatedly that there may well be other contributions to the 800—900 nm absorption, particularly from the heme components [4,11—13]. Although no convincing evidence for this was brought forward, the idea is widely held that baseline absorption, if not a weak absorption band, is contributed by one or both hemes (cf. Ref. 12) in this spectral region.

In a recent paper, largely describing studies of the enzyme by X-ray absorption edge spectroscopy, the near-infrared absorption of cytochrome oxidase was used as an independent check on the valence state of components of the enzyme under different experimental conditions [14]. This work led to the novel proposal that the "total assignment of the 830 nm (or 860 nm) absorption to the copper ... Cu_a, seems inappropriate" and material was presented "in support of ... assignment of considerable absorption to Cu_{a_3}" at 830 nm. From the X-ray data it was argued that Cu_{a_3} is a 'blue' copper ion and would be expected to have corresponding light absorption in the 800 nm region. As pointed out by Powers et al. [14], Buse et al. [15] showed that the amino acid sequence of one of the subunits of cytochrome *c* oxidase has a copper-binding site analogous to that shown to be present in plastocyanin [16], a 'blue' copper protein. Since the totality of these observations and suggestions is of great interest, it occurred to us that EPR and low-temperature optical data (reflectance) recorded by us over the past ten years on hundreds of samples at different and partly intermediate states of oxidation and ligation should have produced some evidence of this if indeed both copper components made a

major contribution to the near-infrared absorption. A survey of these data failed to show such evidence and so did additional experiments carried out following protocols given by Powers et al. [14]. In this report we show examples of the data obtained previously and our recent results. In so doing we would like to emphasize that we are not concerned with the question whether Cu_u has any significant detectable light absorption, possibly in some unusual state of the enzyme; we address ourselves to the question, as brought up in the publication referred to above [14]: does Cu_u absorb light in the 800—900 nm region to an extent that this absorption becomes analytically useful in determining its oxidation state or changes in ligation.

Our work relies heavily on a correlation of low-temperature EPR and optical reflectance spectra recorded on the very same samples of the enzyme. While EPR data on the copper signal of cytochrome oxidase can be obtained with a precision of at least ±5%, reflectance spectroscopy of frozen samples does not provide such precision. Fortunately, because of low scattering losses, the near-infrared region is the most favorable one for obtaining reflectance spectra useful for quantitative evaluation, particularly if the concentration of the absorber is low (cf. Ref. 17). These spectra are far superior in signal to noise to ambient temperature spectra in the near-infrared region. We also had the advantage that, with the large number of spectra available, random errors have been largely eliminated.

Materials and Methods

These were as described in our previous publications on cytochrome oxidase [8,18—21]. Experimental details are given in tables and figure legends. For the experiments shown in Figs. 1—4, 0.2 mM cytochrome oxidase (total heme *a* basis) in 0.01 M Tricine/cacodylate, 0.2% (w/v) Tween 20 of pH 7.4 was used. In the samples derived from rapid-mixing experiments the enzyme concentration reached a similar level by dilution through mixing with an equal volume of reactant and by freezing in isopentane. In all experiments considered here, reflectance spectroscopy was carried out similar to the procedure described [17] but using an EPR sample dewar to hold the sample tubes at the sample port of the integrating sphere. The samples were cooled to —172°C by a flow of cold gaseous nitrogen. For the spectra in the 400—800 nm region a RCA 6217 photomultiplier was used; this was replaced by a RCA 7102 red-sensitive photomultiplier for the 500—1100 nm region. Scanning was started at 800 or 1100 nm, respectively. Spectra in the infrared region were superimposed at 1100 nm, on the assumption that no specific absorption occurred at this wavelength. For the infrared region the spectrum of a fully reduced sample (NADH + phenazinemethosulfate) was taken as a baseline. We find that repeated spectra of individual samples strictly coincide though spectra obtained from various samples with identical contents and treatment may differ slightly in their baseline. Thus different samples of the resting enzyme (i.e as obtained on purification) and the mixed valence enzyme [12] showed baseline differences amounting to approx. 15% of the maximal differences in absorption between each sample and the baseline provided by the spectrum of the reduced sample. With the Cary Model 14 spectrophotometer available, when operated

with a photomultiplier, there was no way of correcting the baseline via the multiplots at wavelengths greater than 800 nm and consequently the spectra beyond 800 nm have a curved baseline. However, since this baseline is reproducible, the curvature does not interfere with an evaluation of the data. Spectra of the 400—800 nm region were superimposed by matching the experimental traces at the trough at 700 nm.

Results

Table I shows results obtained on samples prepared by rapid mixing of reduced cytochrome oxidase with oxidants together with the corresponding controls. When available, measurements on the resting enzyme were used as a basis for comparison. Otherwise the data were expressed as a percentage of the maximum observed. Since under the conditions of the experiment all metal components in the enzyme are expected to be oxidized, the data of Table I (Expt. 1) have no direct bearing on the question of whether Cu_u does absorb significantly in the near-infrared, but the experiment shows the correlation of optical and EPR spectra that can be obtained in our experiments. It should be noted that in all experiments of Table I (not those of Figs. 1—4 and Table II) an additional variable enters the picture: this is the packing into the EPR tube of the sample, rapidly frozen in isopentane. We see from Expt. 1 of Table I that the maximal deviation from the average value is 7.5% for the copper EPR signal and 10% for the optical reflectance spectra. In this experiment we compare essentially fully oxidized samples and thus the question as to whether reflectance spectroscopy produces values proportional to the oxidation state of the copper is not answered. Expts. 2—4, 6 and 7 of Table I as well as Expts. 2 and 3 of Table II, however, provide examples in which partly oxidized samples were also examined. We see a good correspondence between copper signal and absorbance at 800 nm. Understandably, when the observed spectra differ only slightly from the baseline, the scatter of values increases.

We would like to draw particular attention to Expts. 6 and 7 of Table I in which the enzyme had been reduced in the presence or absence of CO and was subsequently reoxidized anaerobically by ferricyanide. Under these conditions one might expect that optical or EPR features of Cu_u might become apparent at early times, either through their presence (Expt. 6) or their absence (Expts. 6 or 7) when compared to samples oxidized by oxygen. Thus, for instance under the conditions of Expt. 6 we observe a large rhombic signal at $g = 6$ accounting for 50—80% of the high-spin heme (cf. Ref. 20) and indicating that there is no interaction between a_3^{3+} and Cu_u. If Cu_u had also become oxidized we would expect to detect at least 50% of it by EPR. On the other hand, in Expt. 7, with CO present, we would assume that both cytochrome a_3 and Cu_u remain reduced [22,23], so that we should have expected to see a diminished absorption at 800 nm if Cu_u contributed significantly to the absorption at this wavelength. Neither of these expectations was realized. A correlation between EPR and optical results similar to that present in Table I is shown in the data from titration experiments assembled in Table II. For this table we selected experiments in which we had addressed ourselves more specifically to the situations also considered in the paper by Powers et al. [14], namely the correlation

TABLE I

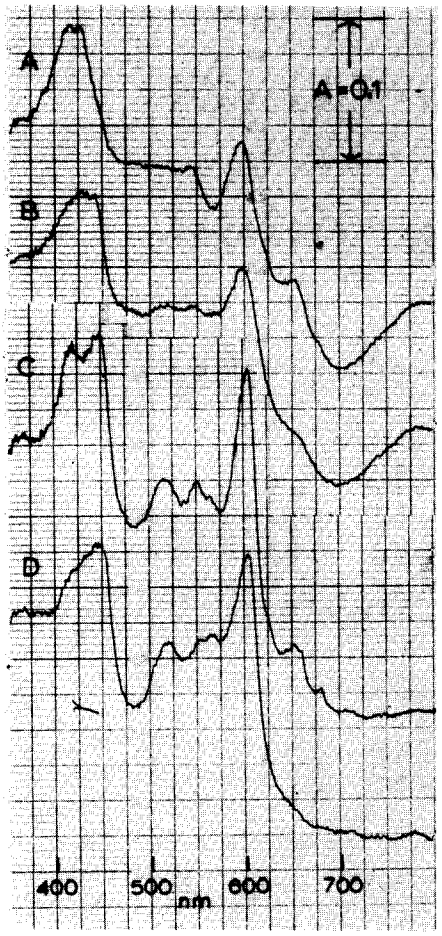
CORRELATION OF EPR SIGNAL AT $g = 2$ AND ABSORBANCE AT 800 nm IN SAMPLES PRODUCED BY THE FREEZE-QUENCH TECHNIQUE

Reduction with NADH was carried out for at least 18 h at 2°C (cf. Ref. 21). The ferricyanide concentration after mixing was between 2 to 6.5 mM. n.d., not determined.

Expt. No.	Sample condition	Addition	Exposure to addition			Copper signal (%)	A_{800} nm (%)
			ms	s	min		
1	Aerobic resting	O_2 buffer	6			100	n.d.
	Anaerobic, reduced by NADH	O_2 buffer	6			111	84
			8			105	94
				1		116	92
				10		106	n.d.
					1	116	100 *
					3.5	104	83
					13.5	109	91
2	Anaerobic, reduced by NADH	N_2 buffer	6			24	20
			6			20.5	17.5
		O_2 buffer	6			90	98.5
			100			89	98.5
				1		100 *	100 *
3	Anaerobic, reduced by NADH	N_2 buffer	6			27.5	24
		O_2 buffer	100			100 *	100 *
		Ferricyanide	6			105	97
			100			110	95
4	Anaerobic, reduced by dithionite	N_2 buffer	6			23.5	14
		Ferricyanide		1.3		91	86
				4.3		100 *	100 *
5	Aerobic, resting	O_2 buffer	6			100	100
	Anaerobic, reduced by ascorbate + cytochrome *c*	O_2 buffer	13			79.5	75
	Anaerobic, reduced by NADH	O_2 buffer	15			82.5	85
6	Aerobic resting	O_2 buffer	6			100 *	n.d.
	Anaerobic, reduced by NADH	N_2 buffer	6			34	23
		Ferricyanide	6			95.5	93
			100			96.5	97
			430			91	100 *
				1.5		95.5	97
7	Anaerobic, reduced by NADH, + CO	N_2 buffer	6			3	13
		O_2 buffer	100			14	22.5
		Ferricyanide	6			79	87
			100			86	98

* When controls of the resting enzyme were not available the percentage signal size was calculated on the basis of the maximal signal observed. When reoxidation by O_2 had been done, the samples so reoxidized were chosen over those reoxidized by ferricyanide.

of the oxidation state of the copper components and near-infrared absorption in CO and formate ('converse') mixed valence states and at states of partial reduction, when two reducing equivalents per 4-metal unit have been added in the presence and absence of CO. One piece of evidence, most emphasized by Powers et al., is the apparent difference in absorption of the oxidized resting enzyme as compared to the enzyme in the mixed valence state [12], i.e. when cytochrome *a* and Cu_d are oxidized, but a_3 and Cu_u remain reduced by virtue



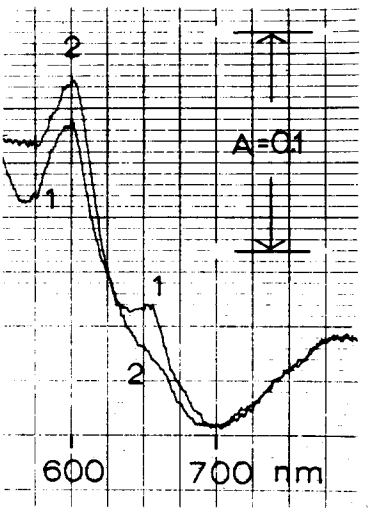


Fig. 1. Low-temperature (—172°C) reflectance spectra between 380 and 800 nm of cytochrome oxidase in the oxidized (A), CO-mixed valence (B), formate-mixed valence (C), and reduced (D) states. 0.2 mM cytochrome *c* oxidase in 0.01 M Tricine/cacodylate buffer of pH 7.4, containing 0.2% (w/v) Tween 20, was made anaerobic by repeated evacuation and flushing with Ar and frozen: A, as it was; B, after reduction by 0.4 mM NADH plus 3 μM phenazinemethosulfate for 15 min in the presence of CO and in the dark, followed by reoxidation with 2 mM ferricyanide for 1 min; C, in the presence of 30 mM sodium formate, 80 μM diaminodurene, after a 30 s exposure to 0.4 mM NADH plus 3 μM phenazinemethosulfate, and D, after reduction by 0.4 mM NADH plus 3 μM phenazinemethosulfate for 15 min.

Fig. 2. Superposition of reflectance spectra of samples analogous to those of Fig. 1A and B except that the sample of the oxidized enzyme (curve 1) was 5—10% more dilute than that of the mixed valence enzyme (curve 2).

of their interaction with CO. (It is generally agreed that both a_3 and Cu_u are reduced in this state, although no direct unambiguous evidence for the state of Cu_u is available [22,23].) The data of Expts. 1 and 2 of Table II and Figs. 1—4 bear on this point. Reflectance spectra of samples from Expt. 2 (Table II) are shown in Fig. 1. If the band height at 800 nm is measured with respect to the trough at 700 nm the difference in absorption is approx. 10% between spectra A and B, the resting and mixed valence [12] states, respectively. Although 10% is within the error of the reflectance technique, we have seen

TABLE II

CORRELATION OF EPR SIGNAL AT $g = 2$ AND ABSORBANCE AT 800 nm IN SAMPLES PRODUCED BY OXIDOREDUCTIVE TITRATION PROCEDURES IN THE PRESENCE AND ABSENCE OF CO

Expt. No.	Sample condition	CO present	Additions	Final concentration of reagent	Time of exposure at 18°C (min)	Copper signal (%)	A_{800} nm (%)
1	Oxidized, resting	—	—			100	100
	Anaerobic, reduced by NADH + PMS, 15 min	—	Ferricyanide	2 mM	1	100	95
		+		2 mM	1	87	90
		—		2 mM	1	98	109
		+		2 mM	1	91.5	100
		+ *		2 mM	1	96.5	93.5
2	Oxidized, resting	—	—			100	100
	Anaerobic reduced by NADH + PMS, 15 min	+	Ferricyanide	2 mM	1	101	89
	Anaerobic	—	NADH + PMS	0.4 mM + 3 μM	15	3	0
	Anaerobic, 30 mM Na^+ formate, 80 μM DAD	—	NADH + PMS	2 mM + 6 μM	0.5	0	0
3	Oxidized, resting	—	—			100	100
	Anaerobic	—	Dithionite	0.5 e^-/heme	10	96.5	98
		—		1 e^-/heme	2	40	54
		+		1 e^-/heme	2	41.5	53
		—		1 e^-/heme	10	46	53
		+		1 e^-/heme	10	51	61.5
		—		1 e^-/heme	170	59	61.5
		+		1 e^-/heme	170	76.5	70.5

* Frozen in collimated beam from Xe lamp.
PMS, phenazinemethosulfate.

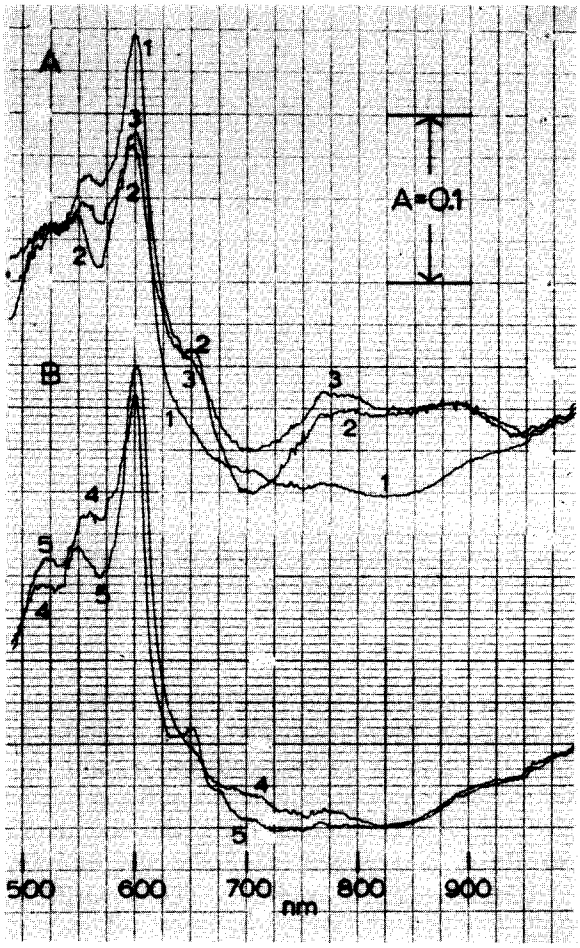


Fig. 3. Superposition of reflectance spectra of the samples of Fig. 1, but from 500 to 1000 nm. In the upper set of curves (A) spectra 1—3 correspond to spectra D (reduced), A (resting), and B (CO mixed valence) of Fig. 1; in the lower set (B) spectra 4 and 5 correspond to spectra D (reduced) and C (formate mixed valence) of Fig. 1.

this difference repeatedly [19] and are inclined to consider it significant. Fig. 2 shows the superposition of spectra from a resting sample (curve 1) and a sample in the (CO) mixed valence state (curve 2). Here we purposely chose samples with equal absorption at 800 nm for superposition so that the shape of the absorption curves in the 700—800 nm region could be better compared. Clearly, there is no significant difference in shape.

A comparison of the reflectance spectra of cytochrome oxidase in the 400—800 nm region suffers from the disadvantage that the baseline is poorly defined, a general difficulty in reflectance spectroscopy. In spectra recorded in this region we therefore made the assumption that the absorption at the minimum at 700 nm was closely similar in the samples to be compared. However, to obviate objections to this, we also recorded spectra from which a long-wavelength baseline can be obtained, as will be shown in Figs. 3 and 4. Before

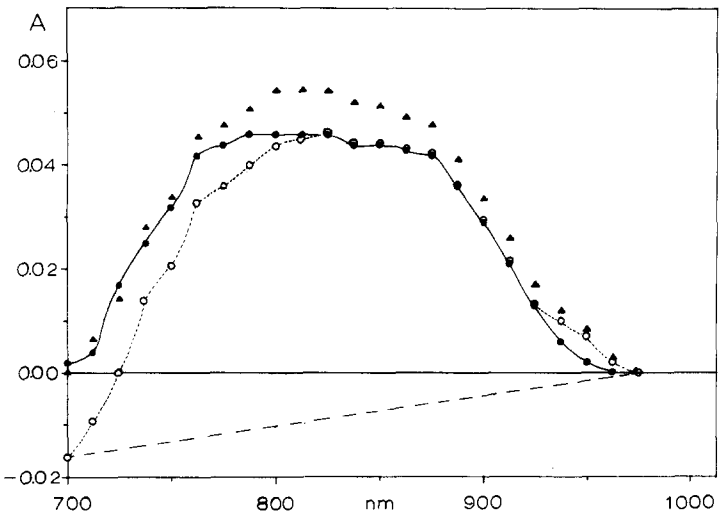


Fig. 4. Difference spectra calculated from a set of spectra analogous to those of Fig. 3 for resting oxidized enzyme minus reduced enzyme (○) and CO-mixed valence enzyme minus reduced enzyme (●). When a linear slope of the baseline is assumed for the spectrum of the resting enzyme and a correction is applied for such a slope, the spectrum would have the slope indicated by (▲).

proceeding to these data, we will consider spectra C and D of Fig. 1, D showing the fully reduced form, and C the so-called 'formate-mixed valence' state; in this state cytochrome *a* and Cu_d are thought to be reduced and a_3 and Cu_u oxidized. Since formate is a weaker ligand than CO, this latter state is not as well defined as the CO-mixed valence state. It appears from the spectrum, though, that at least part of cytochrome a_3 is oxidized (cf. Soret and 655-nm bands); however, no direct information on Cu_u is available. The absorption between 700 and 800 nm is certainly minimal and probably does not exceed the experimental error.

Fig. 3 shows spectra obtained on the same samples used for Fig. 1 but taken with a red-sensitive photomultiplier. The spectra were superimposed in the 1000—1100 nm region where there appeared to be insignificant absorption. Spectra 1 and 4 represent the reduced enzyme, spectrum 2 the resting, oxidized sample, and spectra 3 and 5 the CO- and formate-mixed valence states, respectively. Although it appears that the different curves lie on baselines of somewhat different slope (cf. Materials and Methods) it should be remembered that this slope occurs over a span of 500 nm. This may be due to minor differences in geometry or surface features of the frozen samples and almost certainly has nothing to do with the intrinsic absorbances of the frozen solutions. Despite this shortcoming, the spectra lead to the same conclusion as those of Fig. 1, namely, that the differences in absorption between 800 and 900 nm of the CO-mixed valence state and the resting enzyme on one hand, and the formate mixed valence state and the reduced enzyme on the other, are minimal and certainly not useful for diagnosing the state of Cu_u under these conditions.

The difference in absorption in a set of samples similar to that of Fig. 1A, B and D and Fig. 3A was plotted in the form of difference spectra: resting minus reduced (○) and mixed valence (CO) minus reduced (●). By assuming

that the baseline for the former sample had a linear slope as shown (- - - - -) and applying a corresponding correction, we obtained the spectrum indicated by the symbols ▲. Although this correction procedure may not be entirely satisfactory, the shape and similarity of the absorptions is such that one would have to produce spectra of substantially better quality, if the absorption of another component such as Cu_u were to be determined.

Expt. 3 of Table II is concerned with another point also brought up by Powers et al. [14], namely the electron distribution in samples containing two electrons per four metal centers. These authors state that according to Wever et al. [23] "in the presence or absence of CO . . . the heme *a* and copper of cytochrome a_3 are completely reduced by two electron equivalents per cytochrome oxidase molecule". This is neither compatible with data we have obtained through the years nor does Ref. 23 imply this (see Discussion). Although the extent of reduction of various components depends on the reductant used, the temperature, and the time allowed for electron redistribution, we have under these conditions never seen a clearcut dichotomy between reduction of *a* and Cu_d vs. a_3, even after hours, and certainly not in the absence of CO. At long times with CO the state described by the authors is approached. The data of Expt. 3 (Table II) pertain to this. Again, in these experiments, the parallel behavior of the copper signal and the 800 nm absorption is evident. It is also evident that electron redistribution from Cu_d and cytochrome *a* (cf. Ref. 23) toward cytochrome a_3 and Cu_u is a slow process when only a limiting quantity of reducing equivalents is supplied, and that this redistribution is favored by the presence of CO. However, the situation, with *a* and Cu_d fully oxidized and a_3 and Cu_u completely reduced is rarely, if ever, encountered with one reducing equivalent per heme, even with CO present.

Discussion

It may be appropriate to restate here the aims of this paper: we want to show that at the present state of our knowledge and of published work by other authors [4,5,8,10,12,13,24,25] it is not feasible to determine the oxidation state of the EPR-undetectable copper, Cu_u, from spectrophotometry in the 700—900 nm region. We base this attempt on many years of experimentation and spectroscopy on cytochrome oxidase, both optical at room and low temperature and EPR. We do not mean to say that Cu_u does not absorb light in this spectral region, but such absorption would be within the limits of error of that associated with the determination of the EPR-detectable copper, Cu_d, in all states of the enzyme normally encountered. Nor do we imply that some unusual state of the enzyme may not be produced in which such minor absorption might be observed and exploited, but to our knowledge such a state has yet to be obtained and defined.

One of the critical issues centers around the near-infrared absorption of the enzyme in the mixed valence state (CO). This state has been described and investigated by a number of workers, originally by Horie and Morrison [26], Wharton [5] and by Greenwood et al. [12]. These authors compare room temperature absorption spectra of the resting and mixed valence enzyme in the near-infrared region. Their results agree with ours (cf. Figs. 1 and 2) insofar

as there is no significant change in the observed absorption band as such, if one compares the difference between the trough at 700 nm with the maximum of approx. 830 nm. However, Greenwood et al. [12] observed consistently that the baseline absorption was decreased significantly in the mixed valence state "possibly suggestive of a heme contribution in this region". Neither Wharton [5] nor we observed this difference but reflectance spectroscopy, as we use it, is not well suited to demonstrate changes in baseline. In support of their claim that cytochrome oxidase in the mixed valence state (CO) has considerably less absorption at 800—900 nm than the resting enzyme Powers et al. [14] refer to earlier results obtained by Wever et al. [23], who indeed describe diminished absorption in this spectral region in samples to which one reducing equivalent per heme had been added in the presence of CO. However, EPR spectra of these samples demonstrate that Cu_d is approx. 50% reduced, a situation clearly referred to in the text of Ref. 23. Analogous results were obtained by Babcock et al. [24]. So the data of Ref. 23 are not in disagreement with ours; neither do the authors of Ref. 23 disagree with our conclusions that there is no evidence for a major contribution of Cu_u to the 800 nm absorption. (Wever, R., personal communication). It is also pertinent to mention very recent work supporting our conclusions. In reductive titrations monitored by light absorption, magnetic circular dichroism and EPR spectroscopies, Babcock et al. [24] found good correlation between absorbance at approx. 800 nm and the EPR signal at $g = 2$ and Weintraub [25] followed these features during removal of copper from the enzyme by cyanide at pH $\approx$ 9 or bathocuproine disulfonate at pH $\approx$ 5 [25]. The author concludes that: "Analysis of the near-infrared band at 830 nm revealed that changes in absorbance closely matched changes in the amount of EPR-detectable copper. No visible or near-infrared band was observed which could be directly associated with the EPR-undetectable copper".

We are well aware of the difficulty of obtaining unambiguous results with an enzyme of the complexity of cytochrome oxidase. We would therefore like to point out an assumption which is made in the evaluation of the data and the discussion above; and there is a still a finite chance that this assumption is not correct: It is generally agreed [22,23], and we accept this, that in the mixed valence state both cytochrome a_3 and Cu_u remain reduced in the presence of CO. This state can be generated in a number of ways, all of which are thought to lead to an identical product. However, there is no direct and unequivocal proof that Cu_u is indeed reduced in this state, particularly when it is generated in the presence of an excess of ferricyanide. If Cu_u should be in the oxidized state, then, of course, one would expect the 800—900 nm absorption to be the same as in the resting enzyme, even if Cu_u were a major contributor to the near-infrared absorption. Since the experiments of Wharton [5], Greenwood et al. [12] and Wever (Wever, R., personal communication) as well as our own have shown that the 800—900 nm absorption is not significantly changed on going from the resting to the mixed valence state, we conclude that either Cu_u does not significantly absorb in that region or that Cu_u is indeed not reduced; in other words, the reduction of Cu_u in the mixed valence state (CO) and the attribution to Cu_u of considerable absorption at 800—900 nm are not compatible. This seems to us to be one of the unambiguous conclusions that can

be drawn both from our work and that of others (Refs. 5, 12 and Wever, R., personal communication).

Since most of us believe that Cu_u is indeed largely reduced in the mixed valence state we are faced with the dilemma that the copper component, which according to Ref. 14 may be a 'blue' copper, has but weak absorption at 830 nm ($\epsilon \leqslant 0.3\ mM^{-1} \cdot cm^{-1}$) whereas the EPR-detectable copper Cu_d, contributes greater than or equal to 85% of the absorption at this wavelength. Cu_d however, does not qualify as a 'blue' type copper [14] and is therefore not expected to have a strong S^- to Cu^{2+} charge transfer band in the near-infrared. The EPR signal of Cu_d is in fact thought by some investigators to be due to a sulfur radical interacting with Cu^+ [1,2]. It has been suggested that the absorption observed could then be due to charge transfer from Cu^+ to $S^\cdot$.

Of considerable interest with respect of the present discussion are the amino acid sequence studies on the enzyme carried out by Buse et al. [15], who report a sequence typical of a 'blue' copper site in subunit II. There is little doubt that such a sequence would bind a copper ion under any but the most stringently copper-free conditions. According to Winter et al. [27], one of the large hydrophobic subunits contains both heme and copper when mild methods of isolation are used. However, although these results are of considerable interest in the context of our paper and that of Powers et al. [14], there is no clearcut evidence form the work cited [15,27] which of the two copper components, if not both, reside in the peptides investigated by the respective authors.

On the basis of physical considerations and spectra previously published Powers et al. [14] make estimates of the absorptivities of the chromophore(s) which give(s) rise to the 830 nm band in cytochrome oxidase and of a typical 'blue' copper site at that wavelength. The chromophores of the enzyme should show a combined $\epsilon \approx 2\ mM^{-1} \cdot cm^{-1}$ [23] while for a blue copper site $\epsilon \approx 0.3$—$3\ mM^{-1} \cdot cm^{-1}$ is given [14]. Thus, if we assume that the 'blue' copper in cytochrome oxidase is at the lower limit of this range, absorbing with $\epsilon \approx 0.3\ mM^{-1} \cdot cm^{-1}$, this would be approx. 15% of the total absorptivity of $2\ mM^{-1} \cdot cm^{-1}$ exhibited by the oxidized resting enzyme at 830 nm, which is not incompatible with our data, but certainly not practically useful for analytical purposes at this time, one of the points we wanted to make in this paper.

While we, in the present paper, are stressing these practical aspects, concerning the absorption band centered at 830 nm, data, independent of spectrophotometry in the near-infrared and of EPR, were recently reported which argue against the notion that there is a blue copper in cytochrome oxidase [28,29].

Acknowledgements

This work was supported by a research grant (GM-12394), a Research Career Award (5-KO6-GM-18492) to H.B., and a National Research Service Award (5 F32 GM-05772) to R.W.S. from the Institute of General Medical Services, National Institutes of Health, U.S.P.H.S. C.R.H. was an Established Investigator of the American Heart Association while participating in experimental work reported here.

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