Potentiometric and spectral studies with the two-subunit cytochrome aa_3 from *Paracoccus denitrificans*

Comparison with the 13-subunit beef heart enzyme

Komanduri Pardhasaradhi,* Bernd Ludwig,‡ and Richard W. Hendler*

*Laboratory of Cell Biology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892

‡Institute of Biochemistry, Medical University of Lubeck, Lubeck, Federal Republic of Germany

ABSTRACT Previous work from this laboratory has revealed a complex and interactive redox behavior for the active metal centers in beef heart cytochrome aa_3. All of these centers are contained in two of the 13 subunits which make up the enzyme. The isolated cytochrome aa_3 of *Paracoccus denitrificans* contains only two subunits. The purpose of the current investigation was to see if the complex redox behavior is dependent on the presence of the additional 11 peptides that are present in the mammalian enzyme. In this paper we report that the structurally simpler bacterial enzyme displays a redox behavior which is very similar to that seen with the mammalian enzyme. Therefore, the observed redox behavior does not depend on interactions involving the additional peptides.

INTRODUCTION

In a series of studies of the mammalian cytochrome aa_3, using new techniques of voltage control, and data collection and analysis, a rather complicated and interactive redox behavior was seen. Three forms of cytochrome a_3 were identified. One of these has a high E_m, estimated at ~770 mV (Hendler and Sidhu, 1988). The two other forms have E_m values of ~175 and ~200 mV (Sidhu and Hendler, 1990). These latter two forms are distinguished by their spectra, E_m values and the E_m values of their respective CO-complexes. One of the two appears to be low spin and membrane-associated (Sidhu and Hendler, 1990). Our working hypothesis is that the redox potential of cytochrome a_3, is cooperatively controlled by the redox state of another redox center (Hendler et al., 1986, 1990; Hendler and Westerhoff, in preparation). Cytochrome *a* is also present in more than one form (Hendler et al., 1986; this paper). In addition to cooperative interactions between and among different redox centers, the multiplicity of midpoint potentials can be attributed to different conformational and aggregation states of the enzyme.

The mammalian cytochrome aa_3 has 13 polypeptide subunits (Kadenbach et al., 1986). A cytochrome aa_3 with only two polypeptide subunits has been isolated from *Paracoccus denitrificans* (Ludwig and Schatz, 1980) and this enzyme is similar in many respects to the mammalian enzyme (Ludwig, 1987). If only two subunits can carry the essential redox sites for the enzyme, we wondered whether, perhaps, the additional subunits of the mammalian enzyme were responsible for it's complicated redox behavior. If, on the other hand, the two subunit enzyme shows the same type of interactive redox behavior as the 13 subunit mammalian enzyme, it may represent a minimal structure and redox cooperativity that is required for the energy transduction process. Accordingly, in this paper, we report on the spectral, potentiometric properties of the simpler enzyme in relation to that of the structurally more complicated mammalian enzyme. What we find is a very similar redox behavior for the two enzymes. In the accompanying paper we report on the energy transducing capabilities of the two enzymes in liposomes.

EXPERIMENTAL PROCEDURES

General

The chemical sources, titration procedures, apparatus, and general procedures have been described previously (Reddy and Hendler, 1986; Reddy et al., 1986; Hendler et al., 1986). The *P. denitrificans* cytochrome aa_3 was isolated as previously described (Ludwig and Schatz, 1980) and further purified on an affinity chromatography on a cytochrome *c* column (Bill et al., 1980). The preparation contained ~30 nmol heme *A* per mg protein and ~12 mg protein per ml. The concentrated enzyme was distributed in 50-μl aliquots and stored at −80°C. The beef heart cytochrome aa_3 was prepared by the method of Yoshikawa et al. (1977). The concentrated preparation contained 10–11 nmol heme *A* per mg protein and ~90 mg protein per ml and was stored in 22-μl aliquots at −80°C.

Metal analyses for the 2-subunit *P. denitrificans* and the beef heart cytochrome aa_3 enzymes were carried out in the same laboratory using inductively coupled plasma emission spectroscopy (Steffens et al., 1987). The Cu/Fe

0006-3495/91/08/408/07 $2.00

ratio for both enzymes was ½. The beef heart enzyme had, in addition, 1 Mg and 1 Mn per monomer whereas the bacterial enzyme had ~0.3 Zn, ~0.7 Mg, and ~0.2 Mn per monomer.

Titration protocol for forming low potential cytochrome a_3 with a Soret peak near 430 nm and an alpha peak near 602 nm (a_{3L} [430/602])

This procedure is the same as the one that is effective with the mammalian cytochrome aa_3 (Sidhu and Hendler, 1990). A brief description follows. The bacterial enzyme (6 μM heme *A*, 0.6 mg protein) was added to an aqueous medium containing 125 mM KCl, 62.5 mM potassium phosphate (pH = 7.0) and redox mediators: phanazine methosulfate, 1,2-naphthoquinone, and quinhydrone at 0.1 mM each; potassium ferricyanide (0.2 mM), and chicken whole egg homogenate (~13 mg protein) in a final vol of 3.0 ml. The cuvette was closed and continuously stirred while being flushed under an atmosphere of argon gas for at least 20 min. The voltage of the medium was raised electrically to 400 mV, and then 2–3 μl of 1 M $K_3Fe(CN)_6$ was added, followed by 20 μl (15 mM) diaminodurene. The voltage was held at ~450 mV for 60 min and then electrically reduced to ~95 mV in steps of ~5 mV.

Titration protocol for forming low potential cytochrome a_3 with a Soret peak near 448 nm (a_{3L} [448])

The same procedure as described above for the formation of a_{3L} (430/602) was followed up to the point of flushing the closed cuvette with argon gas for at least 20 min. No additional $K_3Fe(CN)_6$ was added nor was the voltage raised to above 400 mV and held. The voltage of the solution at this point was between 350 and 400 mV. An electrical titration proceeded immediately to ~95 mV in steps of ~5 mV.

Data analysis

All of the spectral data were analyzed by the procedure of singular value decomposition (SVD) as described in our previous papers cited above. For both the mammalian and bacterial enzymes, two redox transitions were seen in which the reduced spectrum for cytochrome *a* rose as that for cytochrome a_3 disappeared. The term "see-saw" was used to describe this phenomenon. In the case of mammalian cytochrome aa_3 the higher of the two "see-saw" transitions involving cytrochrome *a* and cytochrome a_3 was best fit using an *n* value of one. With the bacterial enzyme better fits were generally obtained with an *n* value of two for this and for all of the other titrations encountered. In the interest of enzyme and computer economy, this point was not rigorously examined in the current studies. For the analyses reported in this paper, all analyses were made using *n* values of two.

RESULTS

Titrations of the bacterial enzyme

Fig. 1 shows difference spectra, obtained by SVD analysis of a reductive titration from 380 to 80 mV. The bacterial enzyme was subjected to a full pretreatment, which means that it was exposed to a voltage of ~460 mV in the presence of ~13 mg of fresh egg homogenate for 60 min before the titration which was then conducted in the presence of 1 mM $K_3Fe(CN)_6$, in addition to the usual mediator mix. Four Nernstian redox transitions were seen as shown in the figure. Subsequent titrations under a CO atmosphere (e.g., Fig. 3) showed that the 220 mV component in *a* was due to cytochrome a_3. The important thing to note is that the principal Soret and alpha absorbance features for the cytochrome a_3 transition shown in this panel are located at 430 and 602 nm. The transitions shown in *b* and *c* display a strong Soret peak at 448 nm and an alpha peak at 608 nm. At the same time, a disappearing Soret peak is seen at ~428 nm in *b* and at ~425 nm in *c*. The transition at 367 mV, seen in *d*, shows Soret and alpha peaks at 448 and 607 nm with a deep trough at ~416 nm.

The titration shown in Fig. 2 was conducted from 322 to 89 mV, in the presence of fresh egg homogenate and 0.2 mM $K_3Fe(CN)_6$, but in the absence of a preexposure to high voltage. Titrations under a CO atmosphere (e.g., Fig. 4), identified the transition shown in *a* as cytochrome a_3. The important thing to note here is that the principal Soret peak is at 448 nm instead of at 430 nm as seen in Fig. 1 *a*. A shoulder at 430 nm is evident in the figure. The transitions shown in *b* and *c* are quite similar to the corresponding transitions shown in Fig. 1, *b* and *c*. When attempts were made to start the titration at higher voltages so that the transition at ~367 mV could be seen, the cytochrome a_3 species characterized by a Soret peak at 430 nm became more prominent at the expense of the form with a Soret peak at 448 nm.

When the enzyme was subjected to the full pretreatment as described in the legend to Fig. 1, and the titration then performed under an atmosphere of CO, the results shown in Fig. 3 were obtained. The transition at 220 mV, seen in Fig. 1 *a* with a Soret peak at 430 nm and an alpha peak at 602 nm is not present in Fig. 3. Instead, a different redox transition is seen with an E_m of 269 mV. There is a Soret feature at 435 nm along with an alpha feature at 595 nm evident in *a* and *c*. These are characteristic absorbances for CO complexes of cytochrome a_3 (Yoshikawa et al., 1977; Ludwig and Schatz,

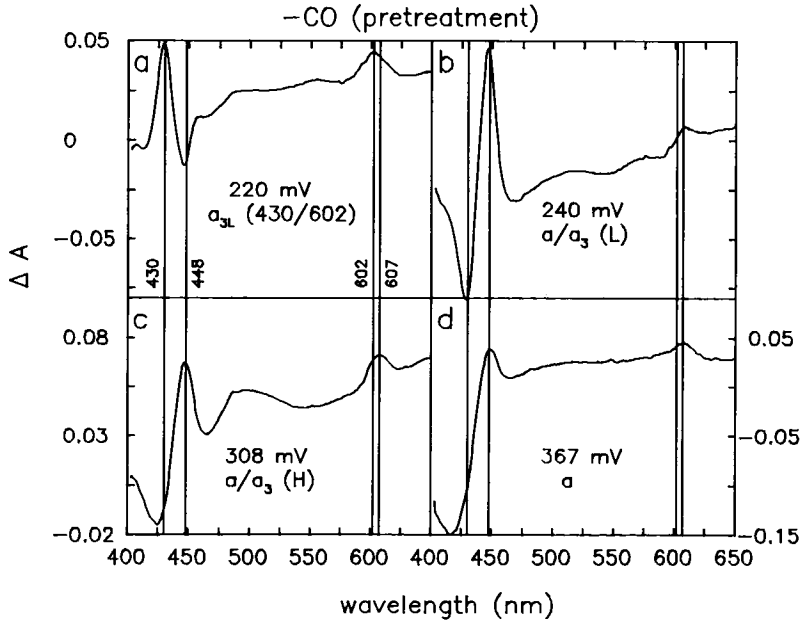

FIGURE 1 *P. denitrificans* cytochrome aa_3 (6 μM heme *A*, 0.6 mg protein) in an aqueous medium containing 125 mM KCl, 62.5 mM potassium phosphate (pH 7.0), hen egg homogenate (13 mg protein), and the following redox mediators: phenazine methosulfate, 1,2-naphthoquinone, and quinhydrone at 0.1 mM each, and potassium ferricyanide at 0.2 mM was made anaerobic by flushing with argon gas for 25 min. The voltage was raised electrically to 400 mV (vs. S.H.E.) and 3 μl (1 M) potassium ferricyanide was added, followed by 20 μl (15 mM) diaminodurene. The voltage was held at ~450 mV for 60 min and then the preparation was subjected to an electrical titration to 95 mV in steps of ~5 mV. All of the collected spectral and voltage data were analyzed by singular value decomposition. The best fits were obtained with *n* values of 2 for all transitions. The spectra shown represent reduced minus oxidized difference spectra for transitions with the E_m values as indicated. The vertical lines are drawn at 430, 448, 602, and 607 nm.

1980). *b* shows essentially the same spectral change as seen for *b* of Figs. 1 and 2, but at a lower E_m. The transition in *d* corresponds to the one shown in *d* of Fig. 1. The transition seen in Fig. 1 *c* is evident in *a* and *c* of Fig. 3, along with the features identified with the CO complex of cytochrome a_3. When the 430 nm species of cytochrome a_3 is liganded to CO, cytochrome *a* appears to be titrated at a somewhat lower voltage. This accounts for finding its features in the transition at 269 mV. We have shown that the two different forms of low potential cytochrome a_3 found in the mammalian enzyme form two different CO complexes (Sidhu and Hendler, 1990). The one formed from the blue-shifted Soret species has a lower E_m than the one formed from the other species. Therefore, we attribute the CO complexes seen in *a* and *c* to the two different forms of cytochrome a_3.

When the enzyme was subjected to less than the full pretreatment, as described in the legend to Fig. 2, and the titration then conducted under a CO atmosphere, results as shown in Fig. 4 were obtained. The transition at 215 mV seen in Fig. 2 *a* is absent under the conditions of this titration. In its place is a new transition at 334 mV with clean Soret and alpha features, characteristic of a cytochrome a_3-CO complex (*a*). The spectral changes seen in *b–d*, correspond to the changes seen in the same panels of the other figures.

Nomenclature

There is a very marked similarity in the spectral, potentiometric species observed in the bacterial and mammalian cytochrome oxidases. To facilitate discussion, we have adopted short descriptive names for the redox components that are seen in the two enzymes.

There is a low-voltage cytochrome a_3 titration that has been separated into two different components using either the bacterial or mammalian enzyme. Based on the location of the Soret and alpha absorbance features and on previously published studies, one of the components appears to be a low spin form, and the other high (Sidhu and Hendler, 1990). The former has a Soret peak at ~428 nm with the mammalian enzyme, and at ~430 nm with the bacterial enzyme. Both of the enzymes display a prominent alpha absorbance at ~602 nm. The individual species are referred to as a_{3L} (428/602) or a_{3L} (430/602). Both of the species are designated, a_{3L} (LS). The other component has its Soret peak at ~446 nm in the mammalian enzyme, and at ~448 nm in the bacterial enzyme. The alpha absorbance is not prominent in

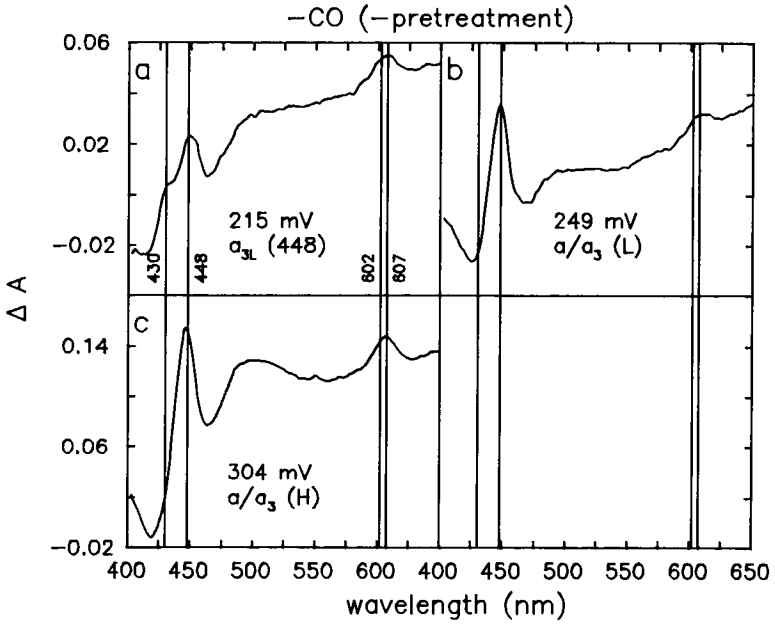

FIGURE 2 The procedure described in the legend to Fig. 1 was followed except for the following. The voltage was not raised above 400 mV nor was it held at its highest level. No additional supplement of potassium ferricyanide was added. Electrical titration was started immediately after the addition of mediators and the establishment of anaerobiosis.

the mammalian enzyme. Although a significant alpha absorbance is seen with the bacterial enzyme, we are not sure at this time whether it is a feature of cytochrome a_3 or whether it is due to an overlap of the cytochrome a spectrum. The individual species are referred to as a_{3L} (448) or a_{3L} (446). Both of the species are referred to as a_{3L} (HS).

In the titration range above that of the various a_{3L}

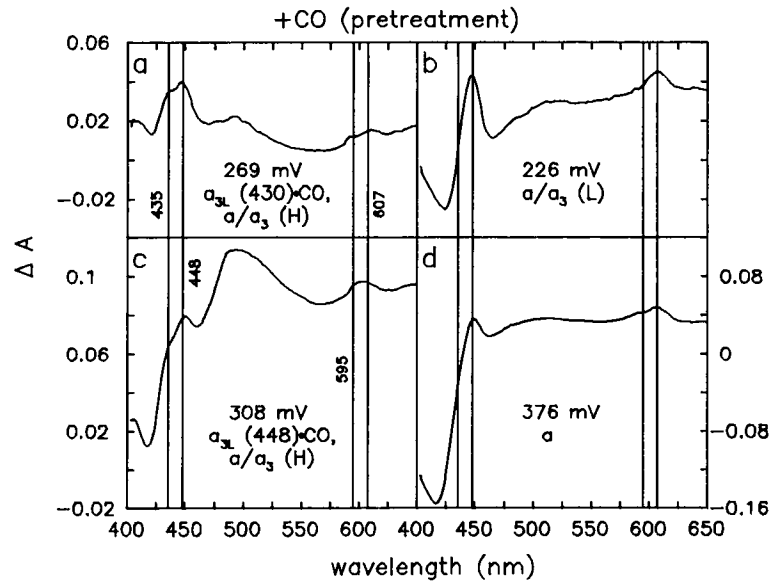

FIGURE 3 The procedure followed was the same as described in the legend to Fig. 1, except for the fact that the argon gas was replaced by CO.

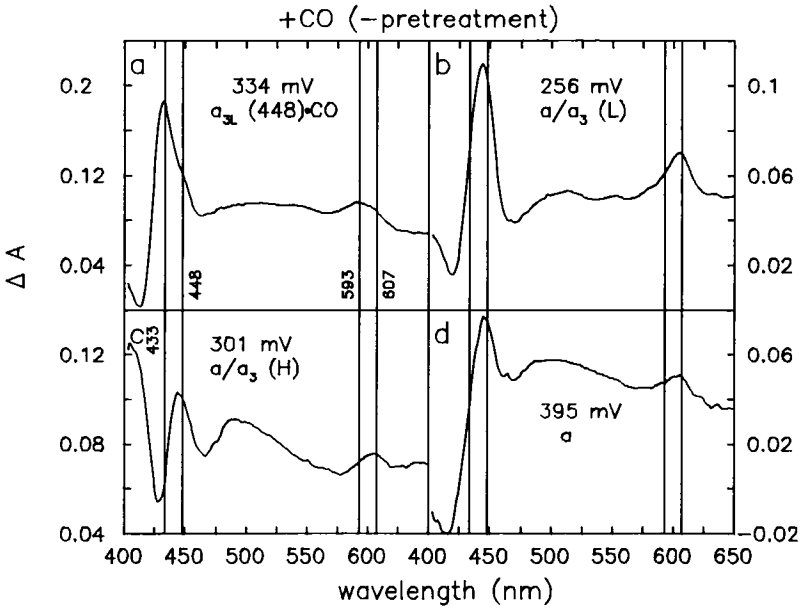

FIGURE 4 The procedure was the same as described in the legend to Fig. 2, except for the fact that the argon gas was replaced by CO.

forms, two individual transitions which show a "see-saw" relationship are seen (Hendler et al., 1986). That is, the Soret peak for cytochrome *a* (at ~447 nm) rises whereas that for cytochrome a_3 (at ~429 nm) falls. We have explained this behavior as due to the simultaneous reduction of cytochrome *a* and oxidation of a high voltage form of cytochrome a_3. The lower potential species of this type is referred to as a/a_3 (L), and the other as a/a_3 (H). Finally, a transition is seen at about 380 mV, which shows only the features of cytochrome *a*, and which is referred to as simply, *a*.

Comparison of the bacterial and mammalian enzymes

The spectral features and E_m's of the major redox species seen in the voltage range of 150 to 400 mV for titrations conducted both under argon and under CO, and using both the bacterial and the mammalian enzyme are summarized in Tables 1 and 2.

The data in Table 1 were obtained for titrations conducted under an argon atmosphere. The lowest E_m species seen with both enzymes that have received the full pretreatment, show a prominent alpha absorbance at 602 nm. The Soret peak is at ~430 nm for the bacterial enzyme and at ~428 nm for the mammalian enzyme. The E_m values for these forms (i.e., a_{3L} [LS]) were 222 and 169 mV, respectively, for the bacterial and mammalian enzymes. In the absence of the full pretreatment, the Soret peaks were seen at 448 and 446 nm, and the E_m values at 221 and 204 mV, respectively, for the bacterial and mammalian enzymes (i.e., a_{3L} [HS] forms).

Both enzymes showed a "see-saw" relationship for the Soret peaks of cytochromes *a* and a_3 in the intermediate voltage range, in the form of two titrating species (i.e., a/a_3 [L] and a/a_3 [H]). The alpha peak for all of these species was seen at ~607 nm, whereas the Soret

TABLE 1 **Comparison of *P. denitrificans* and beef heart cytochrome aa_3's − CO**

	Soret peak			
	Positive	Negative	αpeak	E_m
	nm	*nm*	*nm*	*mV*
a_{3L} (LS)				
Bacterial	430	412, 448	602	222 ± 1 (2)
Mammalian	428	—	602	169 ± 6 (10)
a_{3L} (HS)				
Bacterial	448	413	?	221 ± 6 (2)
Mammalian	446	—	?	204 ± 3 (11)
a/a_3 (L)				
Bacterial	448	428	608	253 ± 11 (5)
Mammalian	446	429	607	260 ± 3 (10)
a/a_3 (H)				
Bacterial	448	425	608	309 ± 4 (5)
Mammalian	446	429	607	340 ± 5 (10)
a				
Bacterial	448	416	608	367 ± 0 (2)
Mammalian	449	415	607	377 ± 0 (2)

TABLE 2 **Comparison of *P. denitrificans* and beef heart cytochrome aa_3's +CO**

	Soret peak			
	Positive	Negative	αpeak	E_m
	nm	*nm*	*nm*	*mV*
a_{3L} (LS) · CO				
Bacterial	435		595	269 (1)
Mammalian	435		595	228 ± 8 (11)
a_{3L} (HS) · CO				
Bacterial	433		595	327 ± 8 (2)
Mammalian	433		595	337 ± 12 (3)
a/a_3 (L)				
Bacterial	448	421	607	250 ± 13 (3)
Mammalian	446	429	607	243 ± 3 (2)
a/a_3 (H)				
Bacterial	448	428	607	305 ± 5 (2)
Mammalian	446	429	607	320 ± 3 (2)
a				
Bacterial	448	416	608	385 ± 10 (2)
Mammalian				

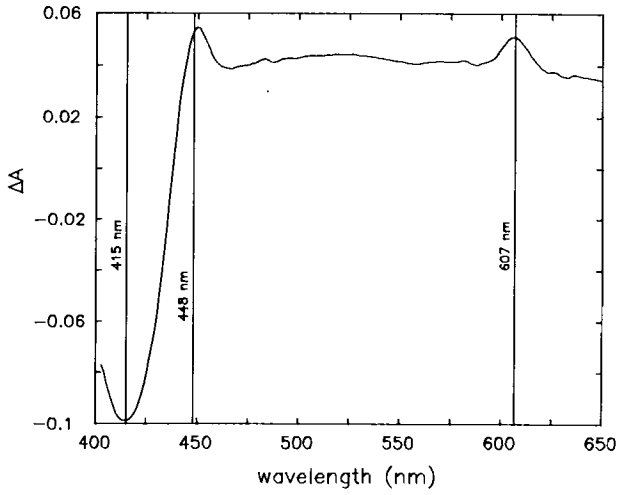

FIGURE 5 The procedure was the same as described in the legend to Fig. 1. However, in this titration, beef heart cytochrome aa_3 was used. The titration range was from 435 to 99 mV. The data were analyzed by SVD. Only the transition at 377 mV is shown.

peaks were at 448 and 446 nm, respectively, for the bacterial and mammalian enzymes. The E_m's were 253 and 260 mV for the a/a_3 (L) forms, and 309 and 340 mV, for the a/a_3 (H) forms, respectively, from the bacterial and mammalian enzymes. A redox transition was seen consistently with the bacterial enzyme at ~370 mV. The spectral change showed a Soret peak at 448 nm, an alpha peak at ~608 nm, along with a very deep trough at ~415 nm. In our earlier studies with the beef heart enzyme, this species was seen irregularly. In view of the results with the bacterial enzyme, we again examined the beef heart enzyme to look for the corresponding titration. Fig. 5 shows that, indeed a similar titration is seen with the mammalian enzyme. The E_m with the mammalian enzyme is at ~377 mV.

The data shown in Table 2 were obtained in titrations conducted under a CO atmosphere. For all of the experiments with both the bacterial and the mammalian enzyme, and whether or not the full pretreatment was used, the lowest E_m component seen in the experiments performed in an Argon atmosphere was absent in the CO titrations. In all cases, the absence of these spectral transitions was compensated by the appearance of new spectral features at ~435 and ~595 nm. These are the characteristic absorption features of CO-complexes of cytochrome a_3. The E_m values for the CO-complexes of the a_{3L} (LS) species were 269 and 228 mV, respectively, for the bacterial and mammalian enzymes, and 327 and 337 mV for the corresponding complexes of the a_{3L} (HS) species. The E_m's and spectral features for the a/a_3 (L), a/a_3 (H), and *a*, species were mostly unaffected by the presence of the CO atmosphere. In the case of the mammalian enzyme, the presence of CO on cytochrome a_3 appears to lower the E_m's of both the a/a_3 species by ~20 mV. This same tendency seems to be present in the bacterial enzyme, when the pretreatment procedure was used, but more experiments are required to quantify the extent of this effect.

DISCUSSION

The question posed in this research is straightforward. Is the redox behavior of the two subunit cytochrome aa_3 simpler than that of the 9–13 subunit mammalian enzyme? The mammalian enzyme has been found to exhibit three redox potentials and spectra for each cytochrome a_3 and cytochrome *a*, and a strong redox cooperativity between cytochrome a_3 and another center. Two of the three redox transitions for mammalian cytochrome a_3 occur below 200 mV (Sidhu and Hendler, 1990). One of these is characterized by a Soret peak at ~428 nm and an alpha peak at 602 nm. The other has a Soret peak at ~446 nm and a weak alpha absorbance near 605 nm. The former appears to be low spin, and its formation is favored by a membrane environment. The third redox transition for cytochrome a_3 occurs near 770 mV. The strong redox cooperative interaction is indicated by the observation that a reductive titration in the voltage range from ~450 to 200 mV causes the simultaneous reduction of cytochrome *a* and *oxidation* of

cytochrome a_3. (Hendler et al., 1986, 1990). This results in a difference spectrum which shows a positive Soret peak for cytochrome *a* at ~446 nm with a negative Soret peak for cytochrome a_3 at ~428 nm. This spectral change was seen in two Nernstian transitions at ~260 and ~340 mV. A third transition for cytochrome *a* is seen at ~380 mV.

The answer to the question posed above is unambiguous. The redox behavior of the two subunit enzyme is every bit as complex as that of the mammalian enzyme.

This leaves unanswered the implicit question behind the experimental one that was posed. What is the role of the additional subunits and the presumed evolutionary advantage of the mammalian enzyme? One possibility, is that the simpler enzyme can not transduce energy as efficiently as its mammalian counterpart. To test this idea we compared the ability of both enzymes to pump protons and to convert respiratory energy into a membrane potential and a delta pH. In the accompanying paper, we report that the bacterial enzyme is every bit as capable as the mammalian enzyme in accomplishing these ends.

A challenge for future research is to define the kind of adaptational and regulation advantage that one suspects must be present in the more evolutionarily advanced and structurally complex enzyme. Some regulational differences between the two enzymes are presented in the accompanying paper.

Part of this research was funded by a grant to Bernd Ludwig from Deutsche Forschung Gemeinschaft (Lu 318/2-1).

Received for publication 1 October 1990 and in final form 23 April 1991.

REFERENCES

Bill, K. B., R. P. Casey, C. Broger, and A. Azzi. 1980. Affinity chromatography purification of ctyochrome *c* oxidase. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 120:248–250.

Hendler, R. W., and G. S. Sidhu. 1988. A new high potential redox transition for cytochrome aa_3. *Biophys. J.* 54:121–133.

Hendler, R. W., G. S. Sidhu, andK. Pardhasaradhi. 1990. High voltage redox properties of cytochrome *c* oxidase. *Biophys. J.* 58:957–967.

Hendler, R. W., K. V. S. Reddy, R. I. Shrager, and W. S. Caughey. 1986. Analysis of the spectra and redox properties of pure cytochrome aa_3. *Biophys. J.* 49:717–729.

Kadenbach, B., A. Stroh, M. Ungibauer, L. Kuhn-Nentwig, U. Buge, and J. Jarausch. 1986. Isozymes of cytochrome *c* oxidase: characterization and isolation from different tissues. *Methods Enzymol.* 126:32–45.

Ludwig, B. 1987. Cytochrome *c* oxidase in prokaryotes. *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Rev.* 46:41–56.

Ludwig, B., and G. Schatz. 1980. A two-subunit cytochrome *c* oxidase (cytochrome aa_3) from *Paracoccus denitrificans*. *Proc. Natl. Acad. Sci. USA.* 77:196–200.

Reddy, K. V. S., and R. W. Hendler. 1986. Complete analysis of the cytochrome components of beef heart mitochondria in terms of spectra and redox properties: the c_1 cytochromes. *Biophys. J.* 49:693–703.

Reddy, K. V. S., R. W. Hendler, and B. Bunow. 1986. Complete analysis of the cytochrome components of beef heart mitochondria in terms of spectra and redox properties. Cytochrome aa_3. *Biophys. J.* 49:705–715.

Sidhu, G. S., and R. W. Hendler. 1990. Characterization of two low E_m forms of cytochrome a_3 and their carbon monoxide complexes in mammalian cytochrome *c* oxidase. *Biophys. J.* 57:1125–1140.

Steffens, G. C. M., R. Biewald, and G. Buse. 1987. Cytochrome *c* oxidase is a three-copper, two-heme-A protein. *Eur. J. Biochem.* 164:295–300.

Yoshikawa, S., M. G. Choc, M. C. O'Toole, and W. S. Caughey. 1977. An infrared study of CO binding to heart cytochrome *c* oxidase and hemoglobin A. *J. Biol. Chem.* 252:5498–5508.