Selective Removal of Subunit VIb Increases the Activity of Cytochrome *c* Oxidase†

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Received March 11, 1992; Revised Manuscript Received September 1, 1992

ABSTRACT: Bovine heart cytochrome *c* oxidase was gel-filtrated on Sephacryl S-300 in 0.05% dodecyl maltoside and in the presence or absence of 1 M KCl. The presence of KCl selectively removed subunit VIb from the enzyme complex, resulting in about doubling of enzymatic activity and an increase of the K_m for ferrocytochrome *c*. In contrast, the proton pumping activity of the enzyme was unchanged. The increase of activity is due to removal of subunit VIb and not of lipids, because titration with asolectin or dodecyl maltoside could not abolish the difference in activity between the 12- and 13-subunit enzyme. Attempts to reconstitute cytochrome *c* oxidase from its separated components were unsuccessful. It is concluded that subunit VIb suppresses the activity of the mammalian enzyme complex by interaction with the active center.

The number of subunits in the cytochrome *c* oxidase (COX)[1] complex increased during evolution from 3 in the bacterium *Paracocccus denitrificans*, to over 7 in the unicellular eucaryote *Dictyostelium discoideum*, to 13 in the mammalian enzyme (Kadenbach et al., 1987b; Ludwig, 1987; Bisson, 1990; Capaldi, 1990b). The three catalytic subunits of the mitochondrial enzyme are homologous to the subunits of bacterial COX, and are encoded on mitochondrial DNA, while the additional subunits are nuclear-coded and partly expressed in a tissue-specific manner (Kadenbach et al., 1987b; Bisson, 1990; Capaldi, 1990a,b). The nuclear-coded subunits were suggested to have a regulatory function (Kadenbach & Merle, 1981; Poyton et al., 1988; Kadenbach, 1986; Bisson, 1990). The regulatory role was substantiated by the opposite effects of intraliposomal ADP and ATP on the kinetics of reconstituted COX from bovine heart (Hüther & Kadenbach, 1987), which are not obtained with the enzyme from *Paracoccus* (Hüther & Kadenbach, 1988) and from bovine liver (Kadenbach et al., 1991). Recently the regulatory role of a nuclear-coded subunit was shown in intact yeast cells. Different rates of electron transfer were measured for COX of yeast cells expressing either the isoform Va or the isoform Vb of the nuclear-coded subunit V (Waterland et al., 1991).

In a recent publication, we have shown that COX from *Paracoccus*, either membrane-bound or dodecyl maltoside-solubilized, has a 4-fold higher activity than the mammalian enzyme (Kadenbach et al., 1991). However, the activity of the mammalian enzyme can be stimulated to the value of the *Paracoccus* enzyme by increasing concentrations of anions in the presence of dodecyl maltoside. Further increase of anion concentrations leads to strong inhibition of both enzymes. The biphasic behavior of activity with increasing anion concentrations is only observed with dodecyl maltoside (Kadenbach, 1986; Reimann et al., 1988; Kadenbach et al., 1987a, 1988). The stimulation of activity at low anion concentrations was assumed to be due to partial and reversible dissociation of nuclear-coded subunits from the catalytic subunits, by the combined action of dodecyl maltoside and anions, comparable to the action of sodium dodecyl sulfate. It was suggested that tight binding of nuclear-coded subunits to the catalytic center suppresses the activity of mammalian COX (Kadenbach et al., 1991).

The removal of nuclear-coded subunits from mammalian COX by detergents and salts, mostly together with the removal of subunit III, has been described repeatedly in the literature [Penttilä et al., 1979; Thompson & Ferguson-Miller, 1983; Thompson et al., 1985; Hill & Robinson, 1986; Gregory & Ferguson-Miller, 1988; see also Capaldi et al. (1983)]. A selective removal of subunit VIb from COX of bovine heart, by gel-filtration in sodium cholate and NaCl, was described by Planques et al. (1989), but no significant influence on the redox and protonmotive activity of the enzyme could be found.

In the present study, we selectively removed subunit VIb by gel-filtration in dodecyl maltoside and KCl and found an increase of activity together with changed kinetics. The data suggest that one role of subunit VIb in the COX enzyme complex could be to suppress the activity.

MATERIALS AND METHODS

Asolectin (L-α-phosphatidylcholine, type II-s from soybean) and cytochrome *c* (type VI from horse heart) were purchased from Sigma. Dodecyl D-maltoside and *N,N,N',N'*-tetramethyl-1,4-phenylenediamine (TMPD) were obtained from Fluka, and Sephacryl S-300 was from Pharmacia. Before use, asolectin was purified by the method of Kagawa and Racker (1971).

Isolation and Subunit Depletion of COX. COX was isolated from bovine heart mitochondria as described previously (Kadenbach et al., 1986). Removal of subunit VIb from the isolated enzyme was performed by gel-filtration on Sephacryl S-300. Two milligrams of COX in about 100 μL was diluted with 200 μL of 10 mM K-Hepes, pH 7.6, 0.05% dodecyl maltoside without (control) or with 1 M KCl (for subunit depletion) and applied on Sephacryl S-300 columns (1.3 × 100 cm) equilibrated with the corresponding buffers as described above. Elution with the same buffers was done for 12 h at 4 °C. Removal of subunit VIb in the presence of 4% cholate and 0.1 M NaCl was done as described by Planques et al. (1989).

Measurement of COX Activity. The activity of COX was determined polarographically as detailed by Büge and Kadenbach (1986). Titration of COX activity with increasing concentrations of anions was performed in 10 mM K-Hepes, pH 7.6, 0.05% dodecyl maltoside, 7 mM Tris–ascorbate, 0.7 mM TMPD, 0.014 mM EDTA, 10 nM COX, and 10 μM

† This paper was supported by the Deutsche Forschungsgemeinschaft (Ka 192/17-5) and the Fonds der Chemischen Industrie.

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[1] Abbreviations: COX, cytochrome *c* oxidase; TMPD, *N,N,N',N'*-tetramethyl-1,4-phenylenediamine dihydrochloride.

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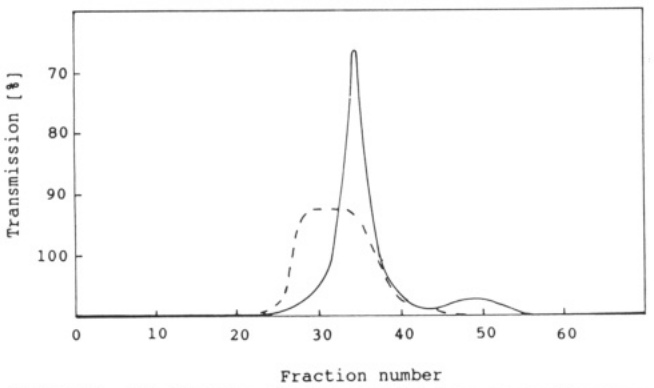

FIGURE 1: Gel-filtration of isolated COX from bovine heart on Sephacryl S-300 in the presence and absence of 1 M KCl. The fraction volume was 1.0 mL. The column (100 × 1.3 cm) was equilibrated and run with 10 mM K-Hepes, pH 7.6, and 0.05% dodecyl maltoside in the absence (- - -) or presence of 1 M KCl (—).

cytochrome *c*. The indicated concentrations of anions were adjusted continuously by adding small volumes of concentrated salt solutions during the polarographic assay. The kinetics of ferrocytochrome *c* oxidation were measured in the same buffer (without addition of salts) at cytochrome *c* concentrations from 0.02 to 40 μM. The titration of COX activity with asolectin (stock solution: 36 mg of asolectin/mL in 10 mM K-Hepes, pH 7.6, and 1% dodecyl maltoside) was done in the same buffer used for the anion titration. Titration of COX activity with detergent was performed by addition of aliquots of 10% dodecyl maltoside to the incubation buffer described for the anion titration. COX activity is presented as turnover number [TN = mol of cytochrome c s^{-1} (mol of heme aa_3)$^{-1}$].

Measurement of Proton Translocation. COX was reconstituted into liposomes by the cholate dialysis method as described by Casey et al. (1979). Proton ejection was measured with a pH microcombination electrode (405-M5 from Ingold, Steinbach) connected to a Beckman Expandometric IV pH meter (Papa et al., 1979). The vesicles were suspended in 1 mM K-Hepes, 100 mM choline chloride, 5 mM KCl, 0.1 mM EDTA, pH 7.0, and 1 μg/mL valinomycin to a final concentration of 0.3–0.5 μM COX. The redox-linked pH changes, elicited by pulses of 3.1–6.3 μM ferrocytochrome *c*, were calibrated with small aliquots of a standard solution of 10 mM HCl.

Attempt To Reconstitute COX from Components. The fraction containing subunit VIb-free COX was divided into two halves and dialyzed either with or without the subunit VIb-containing fraction against the 100-fold volume of 10 mM K-Hepes, pH 7.6, and 0.01% dodecyl maltoside for 4 h and overnight against the same buffer. The dialyzed samples were gel-filtrated on Sephacryl S-300 in 10 mM K-Hepes, pH 7.6, and 0.05% dodecyl maltoside. The fraction containing COX was concentrated on Centricon 30 tubes (Amicon) and separated by SDS–PAGE.

SDS–PAGE. The pooled fractions containing COX were desalted and concentrated by centrifugation in Centricon 30 tubes (Amicon). The fractions containing subunit VIb were precipitated with 0.3 M trichloroacetic acid and dissolved in sample buffer. SDS–PAGE was performed as previously described (Kadenbach et al., 1983).

RESULTS

Gel-filtration of isolated COX from bovine heart on Sephacryl S-300 in 0.05% dodecyl maltoside and the presence or absence of 1 M KCl resulted in the elution patterns shown in Figure 1. The amounts of COX, recovered in the peak

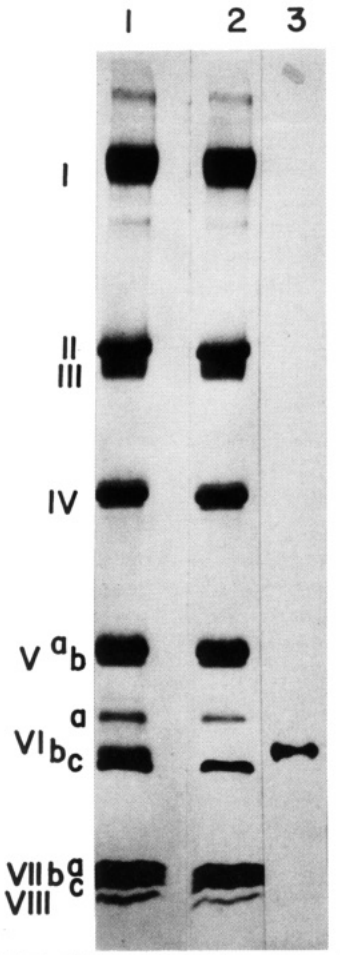

FIGURE 2: SDS–PAGE of COX fractions after gel-filtration in the presence or absence of 1 M KCl. Lane 1, fractions 34–35 of KCl-free treated enzyme; lane 2, fractions 34–35 of KCl-treated enzyme; lane 3, fractions 46–54 of KCl-treated enzyme of the chromatogram of Figure 1.

fractions, were 75%, as determined from the heme aa_3 content. The broad peak obtained in the absence of KCl apparently reflects a mixture of the enzyme in monomeric and multimeric forms. In the presence of KCl, however, a focused peak, reflecting the monomeric enzyme complex (Suarez et al., 1984), and a second small peak of low molecular weight are obtained. The small peak, not obtained in the absence of KCl, represents separated subunit VIb, as follows from the SDS–PAGE of fractions from column chromatography, presented in Figure 2, lane 3. After gel-filtration withoutKCl, the enzyme contains all 13 subunits (Figure 2, lane 1), while treatment with KCl results in an enzyme complex lacking subunit VIb (Figure 2, lane 2), which, however, is recovered in the low molecular weight fraction. Subunit III, which is easily separated by treatment of isolated COX with high detergent and salt concentrations (Penttilä et al., 1979; Thompson & Ferguson-Miller, 1983; Thompson et al., 1985; Hill & Robinson, 1986; Gregory & Ferguson-Miller, 1988; Capaldi et al., 1983), is not detached from COX by gel-filtration in 0.05% dodecyl maltoside and 1 M KCl (Figure 2, lane 2).

A biphasic behavior of activity is obtained when dodecyl maltoside-solubilized COX is titrated with anions (Kadenbach, 1986; Reimann et al., 1988; Kadenbach et al., 1987a, 1988). The initial increase of activity at low anion concentrations was suggested to result from partial and reversible dissociation of nuclear-coded subunits (Kadenbach et al., 1991). In Figure 3, the biphasic change of activity is shown for the KCl-free

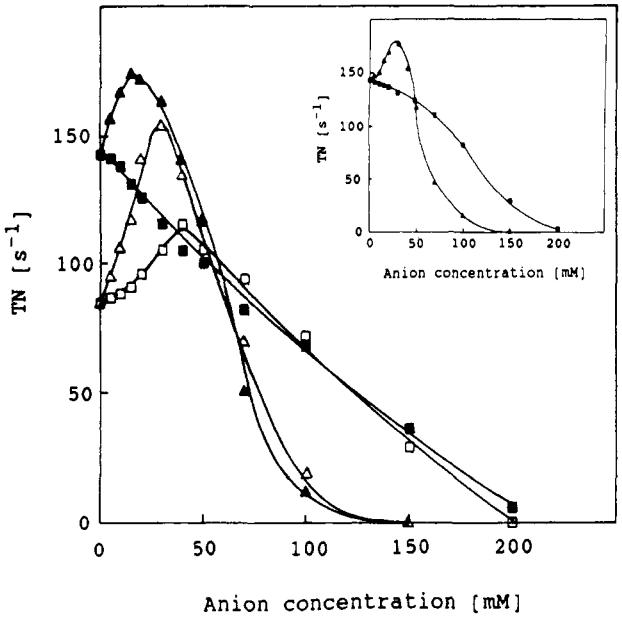

FIGURE 3: Increase of enzymatic activity after gel-filtration of COX in the presence of dodecyl maltoside and KCl. Isolated COX from bovine heart was gel-filtrated on Sephacryl S-300 in 10 mM K-Hepes, pH 7.6, and 0.05% dodecyl maltoside with (■, ▲) or without (□, △) 1 M KCl. Fractions 34–35 of Figure 1 were used for polarographic determinations of activity in 10 mM K-Hepes, pH 7.6, 0.05% dodecyl maltoside, 10 μM cytochrome c, 0.7 mM TMPD, 7 mM ascorbate, 0.014 mM EDTA, and 10 nM COX at the indicated concentrations of KCl (□, ■) or potassium phosphate (△, ▲). Insert: Anion titration of subunit VIb-depleted COX from bovine heart prepared by the procedure of Planques et al. (1989). The titration was done under the same conditions. TN = turnover number.

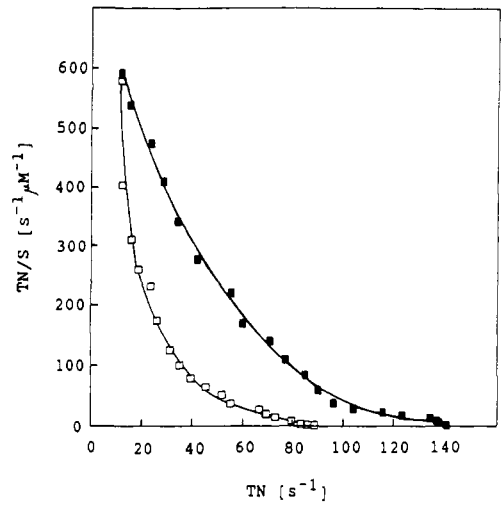

FIGURE 4: Scatchard plot of the kinetics of ferrocytochrome c oxidation by isolated COX after gel-filtration in the presence or absence of 1 M KCl. The polarographic assay of the activity of fractions 34–35 from Figure 1 was performed in the presence of 10 mM K-Hepes, pH 7.6, 0.05% dodecyl maltoside, 7 mM Tris-ascorbate, 0.7 mM TMPD, 0.014 mM EDTA, 10 nM COX, and 0.02–40 μM cytochrome c. TN = turnover number; S = cytochrome c concentration. (□) KCl-free treated COX; (■) KCl-treated COX.

treated enzyme by titration with KCl or with potassium phosphate. Identical curves (i.e., identical TN values) were obtained with fractions 27–28 and 34–35 from the chromatogram of Figure 1 (not shown). In contrast, no stimulation of activity is obtained with the subunit VIb-depleted enzyme, but instead a higher initial activity (170% of control) and a continuous decrease of activity with increasing KCl concentrations are found. Titration with potassium phosphate results in a partial increase of activity at low concentrations with the subunit VIb-depleted enzyme. Further increases of KCl or potassium phosphate concentrations lead to complete inhibition of enzyme activity, which could be explained by competition of salts with the ionic interaction between ferrocytochrome c and COX. The inhibition of COX activity at high ionic strength and in the presence of dodecyl maltoside is completely reversible. This was evidenced by measuring the activity of the 12-subunit and 13-subunit enzymes after preincubation at high salt concentration and subsequent dilution. COX was incubated for 1 h at room temperature in 175 mM potassium phosphate, 10 mM K-Hepes, pH 7.6, and 0.05% dodecyl maltoside. After dilution to 10–50 mM potassium phosphate, identical TN values, as compared to those measured by direct titration (see Figure 3), were obtained for both enzymes (not shown).

These results suggest that at low anion concentrations the bound subunit VIb results in a lower activity as compared to the subunit VIb-depleted enzyme. At higher anion concentrations, no difference in the activity of the two enzymes is found either at different KCl or at different potassium phosphate concentrations. It is assumed that the inhibiting effect of subunit VIb is abolished at high salt concentrations due to loosening of its tight interaction with the catalytic center by dodecyl maltoside and higher salt concentrations. This loosening of binding between subunit VIb and the enzyme complex, however, does not lead to complete dissociation, because dilution of the salt concentration completely reverses the original activity.

In a previous publication by Planques et al. (1989), subunit VIb was separated from bovine heart COX by incubation in 0.1 M NaCl and 4% sodium cholate. We have repeated the procedure of Planques et al. (1989) for the preparation of subunit VIb-depleted COX and obtained the same titration curve with KCl and potassium phosphate in the presence of dodecyl maltoside (see insert of Figure 3).

The influence of subunit VIb removal on the kinetics of COX is presented in the reversed Eadie–Hofstee plot of Figure 4. The polarographic assay was performed with increasing concentrations of cytochrom c in the presence of TMPD. At all cytochrome c concentrations, a higher turnover number (TN) is obtained. The K_m for ferrocytochrome c, which increases with increasing substrate concentrations (Cooper, 1990), is increased at high and decreased at low cytochrome c concentrations after removal of subunit VIb. This result is not in contrast to the result of Planques et al. (1989), who found about the same K_m for the 13-subunit and the 12-subunit enzyme, because at intermediate cytochrome c concentrations we also obtain the same K_m (Figure 4).

It was of interest to see if the proton pumping activity of COX is changed after removal of subunit VIb. We have reconstituted the 12-subunit and the 13-subunit enzyme in liposomes and measured the H^+/e^- stoichiometry as described under Materials and Methods. We obtained H^+/e^- ratios of 0.77 and 0.79 for the 12-subunit and the 13-subunit COX, respectively. These similar values correspond to those obtained by Planques et al. (1989).

In order to prove that the increased activity of COX is due to removal of subunit VIb and not to depletion of lipids, the activities of the two enzymes were titrated with asolectin as shown in Figure 5. Again, biphasic titration curves were obtained, apparently due to the presence of negatively charged lipids in the asolectin preparation (Casey et al., 1982). In fact, no biphasic effects on the activity are obtained by titration with the pure electroneutral phosphatidylcholine (Stroh and Kadenbach, unpublished results). However, under all conditions, the activity of the 12-subunit enzyme has almost twice

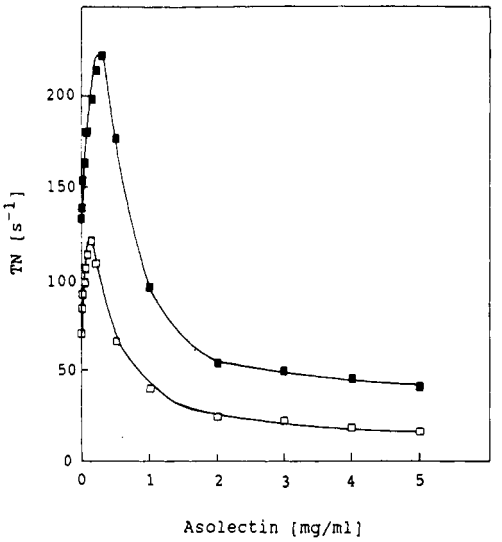

FIGURE 5: Influence of increasing concentrations of asolectin on the activity of control and subunit VIb-depleted COX. The polarographic assay was performed as described under Materials and Methods with increasing concentrations of asolectin as indicated (stock solution, 35 mg of asolectin/mL in 10 mM K-Hepes, pH 7.6, and 1% dodecyl maltoside). TN = turnover number. (□) KCl-free treated COX; (■) KCl-treated COX.

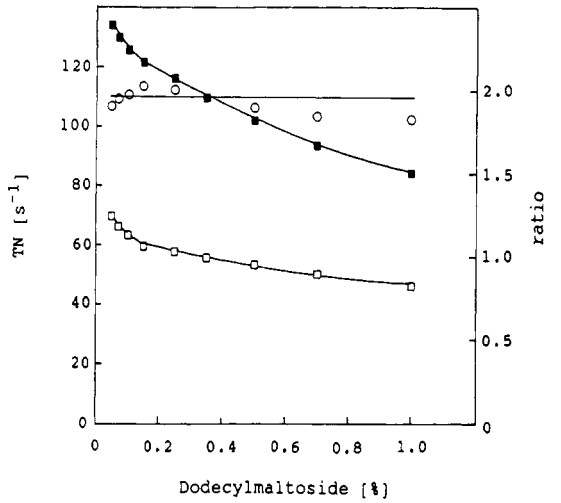

FIGURE 6: Influence of dodecyl maltoside on the activity of control and subunit VIb-depleted COX. Control COX and subunit VIb-depleted COX were obtained by gel-filtration in the absence or presence of 1 M KCl, respectively, and the polarographic assay was performed as described under Materials and Methods with increasing concentrations of dodecyl maltoside as indicated. TN = turnover number. (□) KCl-free treated COX; (■) KCl-treated COX; (○) activity ratio of control and subunit VIb-depleted enzyme.

as high activity as the 13-subunit enzyme (Figure 5). Since the large excess of lipids could replace separated lipids at the subunit VIb-depleted enzyme complex, we conclude that the increased activity of the 12-subunit enzyme is due to removal of subunit VIb and not to a different lipid composition.

This conclusion is further supported by the titration of the two enzymes with dodecyl maltoside, as presented in Figure 6. Increasing concentrations of dodecyl maltoside, present in the polarographic assay medium, decrease the activity of both enzymes, but the subunit VIb-depleted enzyme has about double activity under all dodecyl maltoside concentrations.

In order to prove directly that subunit VIb suppresses the activity of COX, we have attempted to reconstitute the 13-subunit enzyme from its separated components by slow removal of KCl from the mixture of subunit VIb and the 12-subunit enzyme via dialysis. The titration curves of activity with KCl or potassium phosphate of aliquots of the 12-subunit COX, dialyzed either in the presence or in the absence of subunit VIb, did not reveal any difference (not shown). In order to prove physical reassociation, other aliquots of the dialyzed samples were gel-filtrated on Sephacryl S-300. SDS–PAGE of the COX fraction revealed the same pattern of the subunit VIb-depleted enzyme as found before dialysis (see Figure 2, lane 2), indicating that subunit VIb was not reassociated with the enzyme. Thus, it appears that dissociation of subunit VIb from COX by 1 M KCl in the presence of 0.05% dodecyl maltoside cannot be simply reversed by removal of the salt by dialysis.

DISCUSSION

The function of the nuclear-coded subunit VIb of bovine heart COX was investigated by comparing the properties of the 13-subunit and the subunit VIb-depleted enzyme. Both enzymes were treated under identical conditions except for the presence of 1 M KCl during gel-filtration for the depleted enzyme. Removal of subunit VIb from the enzyme complex increases the activity and decreases the affinity to ferrocytochrome *c* for the low-affinity phase (Figure 4). In a previous study by Planques et al. (1989), where subunit VIb was also selectively removed from bovine heart COX by gel-filtration, no increase of activity was obtained. This discrepancy can be explained by the different control used by these authors. While we have gel-filtrated both the depleted and the control enzyme, the 13-subunit enzyme of Planques et al. (1989) was not run on a Sephadex G-150 column, and the detergent for measurement of enzyme activity was not defined. In our hands, the procedure for removal of subunit VIb used by Planques et al. (1989) results in the same stimulated enzyme as obtained by our procedure (see insert of Figure 3).

The direct or indirect involvement of subunit VIb in the interaction of ferrocytochrome *c* with COX is supported by its location on the cytosolic, cytochrome *c* binding site of the transmembranous enzyme complex (Zhang et al., 1988). Together with subunits Va and Vb, subunit VIb does not contain a transmembranous hydrophobic sequence (Kadenbach et al., 1987b), and therefore can be easily removed from the complex. Subunit VIb was also shown to be cross-linked with itself, indicating its location at the interface of the dimeric enzyme complex (Finel, 1987).

The disappearance of the stimulatory phase of activity during KCl titration after removal of subunit VIb (Figure 3) could explain the biphasic anion titration curves of mammalian COX (Kadenbach, 1986; Kadenbach et al., 1987a, 1988, 1991; Reimann et al., 1988), which are not obtained with the *Paracoccus* enzyme (Kadenbach et al., 1988, 1991). Dodecyl maltoside, in cooperation with anions, appears to loosen the tight interaction between nuclear-coded subunits and the catalytic mitochondrial coded subunits, leading to an increase of the down-regulated activity. In fact, the highest activity of isolated mammalian COX is obtained in the presence of dodecyl maltoside (Thompson & Ferguson-Miller, 1983), and this activity corresponds to that of the *Paracoccus* enzyme in the absence of the detergent (Kadenbach et al., 1991). After removal of subunit VIb, titration of the enzyme with potassium phosphate still shows a stimulatory phase, in contrast to titration with KCl (Figure 3). This result could suggest that the 2-fold negatively charged phosphate anion, in contrast to the monovalent chloride anion, dissociates additional subunits, which, however, are not separated from the complex under the applied conditions of gel-filtration.

The failure to reconstitute the 13-subunit COX from its components, i.e., the physical reassociation of subunit VIb

with the 12-subunit enzyme and restoration of the "low activity" COX, could be due to an irreversible conformational change of subunit VIb or the 12-subunit enzyme. This assumption is supported by the involvement of hsp 60 stress proteins (Ellis & van der Vries, 1991; Hartl, 1991) in folding and assembly of mitochondrial proteins in the matrix. These chaperonins maintain the unfolded structure of nuclear-coded proteins before and during assembly into enzyme complexes (Ostermann et al., 1989).

Why should the activity of mammalian COX be down-regulated? We suggested that binding of allosteric effectors to nuclear-coded subunits could regulate (increase) the activity of COX via conformational changes (Kadenbach, 1986). In fact, ADP and ATP have been identified as allosteric effectors of mammalian COX [Hüther & Kadenbach, 1988; Bisson et al., 1987; for a review, see Kadenbach and Reimann (1992)]. A possible allosteric effector, specifically binding to subunit VIb, however, remains to be identified.

ACKNOWLEDGMENT

We thank Roswitha Roller-Müller for careful preparation of the final manuscript.

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