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Tissue-specific regulation of bovine heart cytochrome-*c* oxidase activity by ADP via interaction with subunit VIa

(conformational change/allosteric effector/crossreacting monoclonal antibody/nuclear-coded subunit)

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ABSTRACT **The activity of reconstituted cytochrome-*c* oxidase (EC 1.9.3.1) from bovine heart is stimulated by intraliposomal ADP but not by NaCl of the same ionic strength. A monoclonal antibody which reacts with subunits VIa-H (heart-type) and VIc, due to the evolutionary relationship between these subunits, also stimulates the activity of the enzyme from bovine heart but not from bovine liver. The antibody induces a conformational change in the heart enzyme but not in the liver enzyme, as shown by the visible difference spectrum. Preincubation of heart cytochrome-*c* oxidase with the antibody prevents stimulation of activity by intraliposomal ADP after reconstitution in liposomes. Reconstituted liver cytochrome *c* oxidase is not stimulated by intraliposomal ADP. The data suggest tissue-specific regulation of the activity of cytochrome-*c* oxidase by ADP via interaction with the matrix domain of subunit VIa-H.**

Cytochrome c oxidase (COX), the terminal enzyme of the respiratory chain, is essential for aerobic life of all macroscopic organisms and of some bacteria. During evolution the number of subunits in the enzyme complex increased from 3 in *Paracoccus denitrificans*, to 7 in *Dictyostelium discoideum*, to 9 in yeast, to 13 in mammalian organisms (1–5). The 3 catalytic subunits of eukaryotic COX, which are homologous to the bacterial subunits, are encoded on mitochondrial DNA, while the 10 additional subunits are encoded in the nucleus. Despite the large variability in subunit number almost no differences were observed in the catalytic properties of COX from bovine heart and *Paracoccus* (6–10). In fact, it was debated whether nuclear-coded subunits are essential for the catalytic activity of the enzyme (11–14). Kadenbach and coworkers (1–3, 15) have suggested a regulatory role for nuclear-coded subunits, proposing modulation of the rate of respiration and the efficiency of energy transduction by binding of allosteric effectors and transmission of the signal to the catalytic center via conformational change.

Regulation of COX activity by allosteric effectors has been shown for ATP (16–23) and ADP (21, 22). It was difficult, however, to distinguish between specific allosteric interactions with a subunit and nonspecific ionic effects (24, 25). A specific interaction with a nuclear-coded subunit was assumed for ADP, because intraliposomal ADP decreased the K_m for cytochrome *c* of reconstituted COX from bovine heart but not from *Paracoccus* (22). In recent studies we have demonstrated that ADP interacts tissue-specifically with mammalian COX: in contrast to the heart enzyme, with reconstituted COX from bovine liver no stimulation of the K_m for cytochrome *c* was obtained with intraliposomal ADP (ref. 26; A.R. and B.K., unpublished work).

In the present study a monoclonal antibody to COX subunits VIa and VIc was applied to investigate the effect of intraliposomal ADP on the activity of reconstituted COX from bovine heart and liver. The crossreactivity of the monoclonal antibody with two COX subunits was previously shown to be due to the evolutionary relationship between subunits VIa and VIc (27). The antibody reacts tissue-specifically with the heart isoform of subunit VIa (VIa-H) but reacts equally well with subunits VIc of COX from bovine heart and liver. Here we show that the antibody, like ADP, stimulates the activity of COX from heart but not from liver. In addition, the monoclonal antibody prevents the stimulation of the heart enzyme activity by ADP. The data suggest tissue-specific regulation of COX activity by ADP via specific interaction with the matrix domain of the transmembrane subunit VIa-H.

MATERIALS AND METHODS

COX from bovine heart and liver was prepared from isolated mitochondria (28). The monoclonal antibody to subunits VIa and VIc was prepared and characterized previously (clone 86 of ref. 27). Beef standard IgG was purchased from Bio-Rad. Asolectin (L-α-phosphatidylcholine, type II-s from soybean) and cytochrome *c* (type VI, from horse heart) were obtained from Sigma. Before use, asolectin was purified by the method of Kagawa and Racker (29). Valinomycin and carbonylcyanide *m*-chlorophenylhydrazone (CCCP) were bought from Boehringer Mannheim.

Reconstitution of COX. COX was reconstituted in liposomes by an adsorption method (A. Weishaupt and B.K., unpublished work). Asolectin (40 mg/ml) was sonicated in 1.5% (wt/vol) sodium cholate/10 mM potassium Hepes, pH 7.4/40 mM KCl (20), and COX was added to a final concentration of 3 μM, together with 10 mM ADP or 136 mM NaCl, when indicated. The detergent was removed by adsorption to purified Amberlite XAD-2 (50 mg/ml; Serva) (30) by incubation at 4°C overnight with shaking. Reconstitution in the presence of antibodies was done after preincubation of COX (4 μM) with equimolar amounts of IgG in 10 mM potassium Hepes, pH 7.4/40 mM KCl for 30 min at room temperature. The orientation of COX in the vesicles was calculated from the reduced spectrum obtained by impermeant (ascorbate and cytochrome *c*) and permeant [*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD)] reducing agents in the presence of cyanide (31). Orientation values ranged from 66% to 79% of cytochrome *c* binding sites outside.

Purification of Immunoglobulin. IgG1 was purified from cell culture supernatants (clone 86 of ref. 27) by chromatography on protein A-Sepharose CL-4B (Pharmacia), essentially as described by Ey *et al.* (32), and concentrated by centrifugation in Centricon 30 tubes (Amicon).

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Abbreviations: COX, cytochrome-*c* oxidase; TN, turnover number (mol of ferrocytochrome *c* per second per mol of heme aa_3); TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; subunit VIa-H, heart-type subunit VIa.

Measurement of COX Activity. The activity of isolated COX was measured polarographically with a Clark-type electrode according to Ferguson-Miller *et al.* (33) in 10 mM potassium Hepes, pH 7.4/0.05% dodecyl maltoside/7 mM Tris ascorbate/1 mM TMPD/10 μM cytochrome *c*/20 nM bovine heart or liver COX. The enzymes were preincubated overnight at 4°C either with standard bovine IgG or with purified IgG against COX subunits VIa and VIc in 10 mM potassium Hepes, pH 7.4/0.05% dodecyl maltoside.

The activity of reconstituted COX was measured polarographically in the absence of TMPD in 10 mM potassium Hepes, pH 7.4/40 mM KCl/30 μM EDTA/25 mM Tris ascorbate/20 nM reconstituted COX/0.02–40 μM cytochrome *c* in the presence of valinomycin (1 μg/ml) and CCCP (3 μM) at 25°C. The activity is presented as turnover number (TN, mol of ferrocytochrome *c* per second per mol of heme aa_3).

SDS/PAGE and Western Blot. SDS/PAGE of isolated COX from bovine heart and liver was performed according to either Schägger and von Jagow (34) or Kadenbach *et al.* (35). Blotting of the gels on nitrocellulose and immunostaining were done as described (36).

RESULTS

In previous studies intraliposomal ADP was shown to decrease the K_m for cytochrome *c* of reconstituted COX from bovine heart when measured by the photometric assay (21, 22). No effect of intraliposomal ADP on the kinetics of COX was found with the polarographic method in the presence of TMPD, and it was suggested that intraliposomal ADP affected the rate of dissociation of ferricytochrome *c* (22), because in the photometric assay, but not in the polarographic assay, the dissociation of ferricytochrome *c* is assumed to be the rate-limiting step (33). We have now found that the effect of intraliposomal ADP on the kinetics of reconstituted COX can also be measured with the polarographic method when TMPD is omitted. TMPD was suggested to mediate electron transfer from ascorbate to COX without involving dissociation of ferricytochrome *c* (33). As shown in Fig. 1, intraliposomal ADP stimulates the activity of reconstituted COX from bovine heart at all concentrations of cytochrome *c*, when measured by the polarographic method in the absence of TMPD. The effect of ADP is specific, because intraliposomal NaCl at even higher ionic strength does not affect the activity. The stimulatory effect of ADP is not due to a different orientation of COX in liposomes, because it has been measured repeatedly with preparations of identical orientation. In addition the ADP effect is abolished by addition of TMPD during the polarographic assay (F. Rohdich and B.K., unpublished work). The stimulation by intraliposomal ADP of the activity of reconstituted COX is not obtained in the absence of uncoupler (data not shown). The specificity of the ADP effect will be described in a separate publication. Intraliposomal ATP also stimulates COX activity, but to a lower extent (F. Rohdich and B.K., unpublished work). The effect of ADP is tissue-specific. No influence of intraliposomal ADP on the kinetics of COX from bovine liver was found (F. Rohdich and B.K., unpublished work; see also Fig. 5). COX enzymes from bovine heart and liver differ in subunits VIa, VIIa, and VIII (37, 38).

To investigate the interaction of ADP with a specific COX subunit, we applied a monoclonal antibody that was raised against the bovine heart enzyme and that binds to subunits VIa-H and VIc. The crossreactivity of the antibody was shown to be due to the evolutionary relationship between subunits VIa-H and VIc (27). The reactivity of the antibody with COX from bovine heart and liver is shown in the Western blot of Fig. 2. The monoclonal antibody reacts with subunits VIc of both enzymes but only with subunit VIa of the heart enzyme (lanes 2 and 3). In contrast to subunit VIc, subunit VIa is expressed tissue-specifically in COX from heart and liver (37, 38). While subunit VIa-H gives a small band, a broad band is obtained with subunit VIc. This anomalous behavior of subunit VIc is also found for subunits Vb and VIIc with the gel system of Schägger and von Jagow (34) (lanes 1 and 4) but not with the urea-containing gel system of Kadenbach *et al.* (35) (lanes 5 and 6). However, no

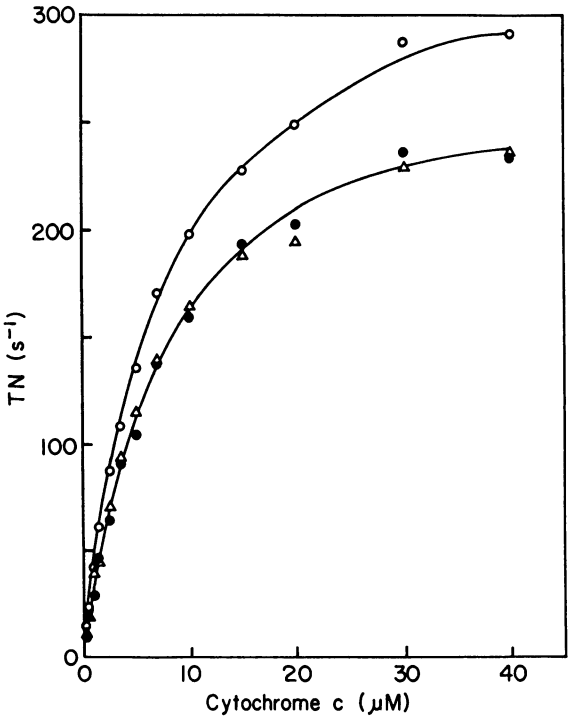

FIG. 1. Effect of intraliposomal ADP on the activity of reconstituted COX from bovine heart. COX was reconstituted in the presence of 10 mM ADP (○), 126 mM NaCl (△), or without additions (●), and the activity was measured polarographically in the absence of TMPD.

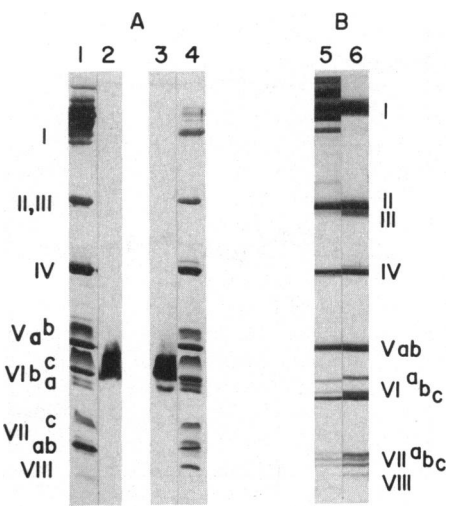

FIG. 2. SDS/PAGE and Western blot analysis of isolated COX (20 μg per lane) from bovine liver (lanes 1, 2, and 5) and heart (lanes 3, 4, and 6). The isolated enzymes were separated either by the method of Schägger and von Jagow (34) (*A*) or by the method of Kadenbach *et al.* (35) (*B*). The gel bands were stained with Coomassie blue (lanes 1 and 4–6) or were blotted onto nitrocellulose, incubated with a monoclonal antibody that recognizes subunits VIa and VIc, and immunostained (lanes 2 and 3).

reaction of the monoclonal antibody with subunit VIa of COX from heart was obtained in a Western blot when the gel system of Kadenbach *et al.* was applied (27).

Titration of isolated COX from bovine heart with the antibody results in increasing activity, as measured polarographically in the presence of TMPD (Fig. 3). Maximal activity is found after addition of stoichiometric amounts of the antibody, while further addition of the purified IgG results in partial decrease of activity, probably due to binding of a second molecule of the antibody to subunit VIc. In contrast to the heart enzyme, no stimulation of activity is obtained with the liver enzyme under identical conditions. This result suggests that the increase of activity of COX from bovine heart is due to binding of the monoclonal antibody to the tissue-specific subunit VIa-H.

Binding of the antibody to subunit VIa-H is associated with a conformational change of the catalytic center of the enzyme, as follows from the experiment shown in Fig. 4. The visible difference spectrum of isolated COX from heart and liver was recorded after addition of the purified IgG against subunit VIac to the sample cuvette and of a control IgG to the reference cuvette. The spectral change of the heart enzyme indicates a broadening of the γ band, whereas no spectral change was obtained with the liver enzyme (Fig. 4), suggesting specific binding of the antibody to subunit VIa-H. This result also indicates that binding of the antibody results in conformational interactions between the nuclear-coded subunit VIa-H and the mitochondrially coded subunit I, which carries the two heme *a* groups (4, 5).

The binding site for ADP appears to be located in close proximity to the binding site for the monoclonal antibody at subunit VIa-H. The two enzymes were preincubated with stoichiometric amounts of either a control IgG or the specific IgG to subunits VIa and VIc and then reconstituted in the

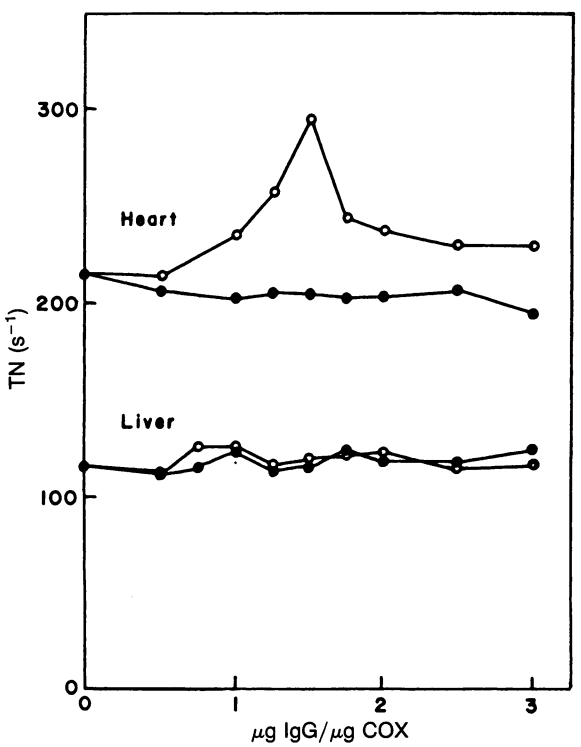

FIG. 3. Titration of isolated COX from bovine heart and liver with a monoclonal antibody that recognizes COX subunits VIa and VIc. Enzymatic activity was measured by the polarographic method in the presence of TMPD. Control IgG (●) or purified IgG from cell culture supernatants of clone 86 (27) (○) was preincubated with COX from bovine heart and liver as indicated up to a molar ratio of 2:1, which corresponds to 3 μg of IgG per μg of COX.

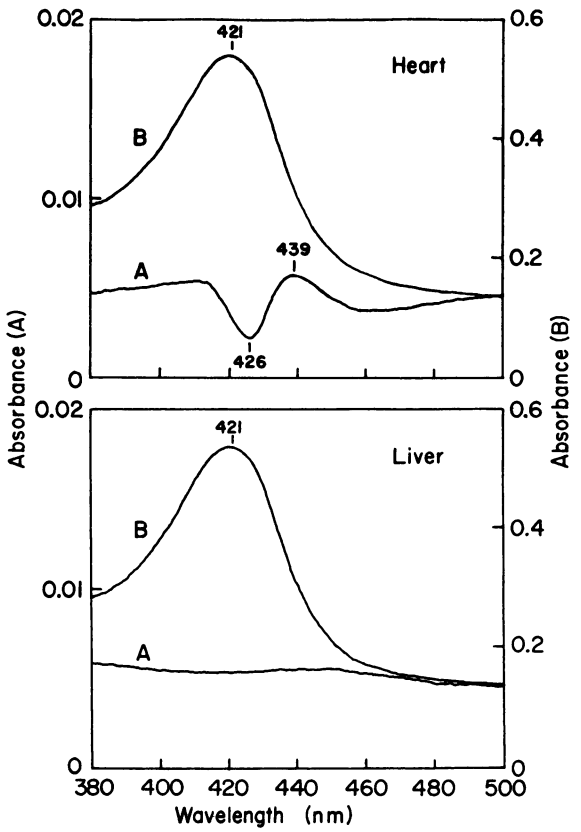

FIG. 4. Influence of a monoclonal antibody that recognizes COX subunits VIa and VIc on the visible spectrum of isolated COX from bovine heart (*Upper*) and liver (*Lower*). Spectra A: difference (left ordinate) spectra of 2 μM isolated COX in 10 mM potassium Hepes, pH 7.4/0.05% dodecyl maltoside, 30 min after addition of 2 μM IgG against subunits VIa and VIc to the sample cuvette, and 2 μM bovine control IgG to the reference cuvette, at room temperature. Spectra B: absolute (right ordinate) spectra of oxidized isolated COX from bovine heart or liver.

presence or absence of ADP. Preincubation of COX from heart with standard IgG did not affect the stimulatory effect of intraliposomal ADP (Fig. 5*A*). From the reversed Eadie–Hofstee plot (Fig. 5*A Inset*) a decreased apparent K_m for cytochrome *c* can be deduced for the low-affinity phase of ferrocytochrome *c* oxidation (see legend to Fig. 5), as previously described (21, 22). In contrast, complete inhibition of the stimulatory effect of intraliposomal ADP was obtained after preincubation of the heart enzyme with the specific antibody (Fig. 5*B*). This preincubation alone resulted in partial increase of activity, which was not further influenced by intraliposomal ADP. In contrast to COX from heart, the activity of reconstituted COX from liver was not affected by preincubation with the specific antibody as compared to preincubation with standard IgG (Fig. 5 *C* and *D*). Furthermore, intraliposomal ADP did not stimulate the activity of the liver enzyme but led to a small inhibition, independent of preincubation of the enzyme with standard IgG or with IgG against subunits VIa and VIc.

DISCUSSION

In this investigation the effect of an allosteric effector (ADP) on the activity of COX is traced back to an interaction with a tissue-specific nuclear-coded subunit (VIa-H) by use of a monoclonal antibody. The interaction of ADP with subunit VIa-H is supported by previous preliminary studies, where a specific binding of 8-azido-[β-^{32}P] ADP to subunit VIa (or VIb) of soluble COX from bovine heart was found (39). The

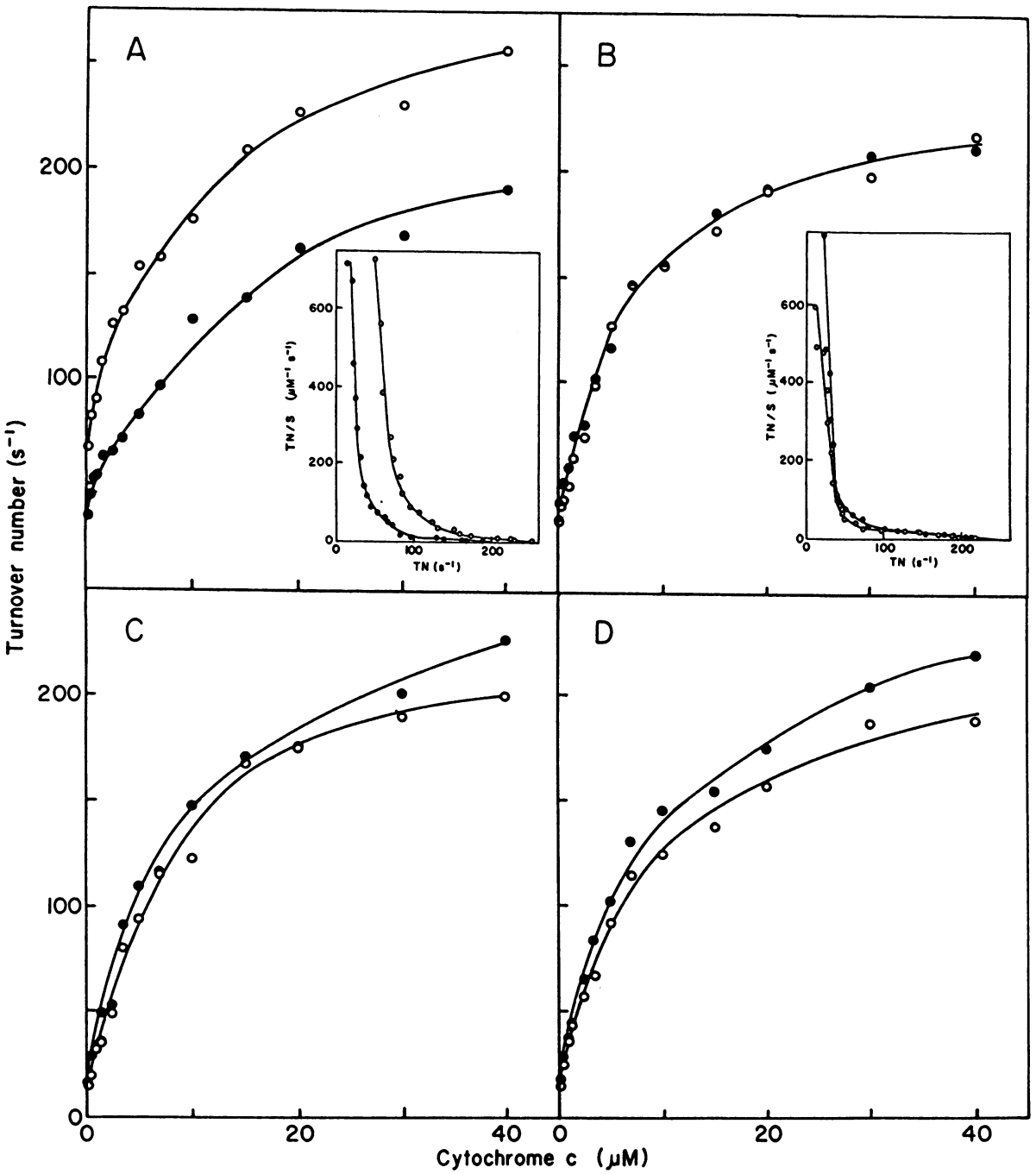

FIG. 5. Effect of intraliposomal ADP and of a monoclonal antibody that recognizes subunits VIa and VIc on the activity of reconstituted COX from bovine heart and liver. COX from bovine heart (*A* and *B*) or bovine liver (*C* and *D*) was reconstituted by the adsorption method in the presence (○) or absence (●) of 10 mM ADP. The enzymes were preincubated either with control IgG (*A* and *C*) or with IgG against COX subunits VIa and VIc (*B* and *D*). After reconstitution the enzymatic activity, was measured polarographically in the presence of CCCP and valinomycin, but without TMPD, at various concentrations of cytochrome *c*. *Insets* of *A* and *B* represent the data in reversed Eadie–Hofstee plots. From the plots the following apparent K_m values can be graphically deduced for the high- and low-affinity phases of ferrocytochrome *c* oxidation. In *A* (control IgG): in the absence of ADP, K_m(high) = 0.02 μM and K_m(low) = 10 μM; in the presence of ADP, K_m(high) = 0.04 μM and K_m(low) = 4 μM. In *B* (anti-subunits VIa and VIc): in the absence of ADP, K_m(high) = 0.02 μM and K_m(low) = 7 μM; in the presence of ADP, K_m(high) = 0.09 μM and K_m(low) = 8 μM.

crossreactivity of the monoclonal antibody with subunits VIc and VIa-H was previously shown to be due to the evolutionary relationship between these subunits (27). The homology between subunits VIc and VIa-H concerns only the N-terminal part, whereas the homology between subunits VIa-H and VIa-L (liver-type) is mainly found in the C-terminal region (Fig. 6). Subunits VIa and VIc of COX from bovine heart contain a transmembrane domain (3), and were shown by Capaldi and coworkers (46, 47) to be oriented within the inner mitochondrial membrane with the N-terminal part directed toward the matrix. Therefore the stimulatory effect of intraliposomal ADP on the activity of reconstituted COX implies interaction with the matrix domain of the enzyme. Since no effect was found with the liver enzyme, we conclude that the site of interaction of intraliposomal ADP with bovine heart COX must be located within the 17 N-terminal amino acids of subunit VIa-H. This sequence is rich in glycine, similar to other nucleotide-binding proteins (43, 44). The

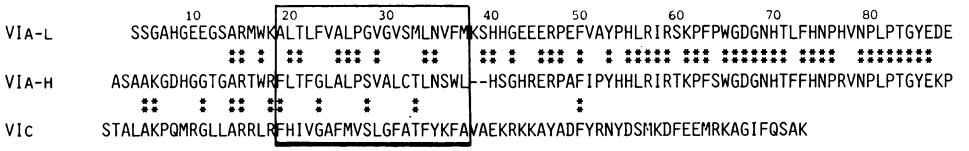

FIG. 6. Alignment of the mature amino acid sequences of subunits VIa-L (liver-specific) VIa-H, and VIc from bovine COX. Sequence homologies are indicated by asterisks, and the transmembrane hydrophobic domains are boxed. Amino acid sequences are from refs. 40 (VIa-L), 41 (VIa-H), and 42 (VIc).

differences in the kinetics of reconstituted COX from bovine liver and heart have been studied in detail previously (45).

The change of the difference spectrum of COX from bovine heart but not from bovine liver (Fig. 4) indicates that the conformational change of the enzyme is due to the binding of the antibody to subunit VIa-H. The maximal stimulation of soluble COX from bovine heart was obtained after addition of the antibody at the molar ratio of 1:1 (Fig. 3), whereas a decrease was found with higher ratios. This result suggests that the antibody has a higher affinity for subunit VIa-H than for VIc of the native enzyme, whereas with the dissociated subunits a higher reactivity to subunit VIc was found (27).

The results of this study are a direct indication of tissue-specific regulation of COX activity by an allosteric effector via interaction with a nuclear-coded subunit, as previously suggested (15). A modulation of COX activity by isoforms of a nuclear-coded subunit was recently shown in intact yeast cells by the groups of Poyton and Chance (48). COX of yeast cells, expressing the subunit Vb isoform, has a higher turnover rate and rate of heme *a* oxidation than COX of yeast cells expressing the subunit Va isoform.

The physiological meaning of stimulation of COX activity by ADP in the matrix compartment appears evident. When the ATP consumption in the heart at high beating rate leads to increased ADP concentrations in the mitochondrial matrix, the respiratory rate, and consequently ATP synthesis, increases independently of respiratory control. This regulatory mechanism in COX of the heart may be essential for survival of the individual, whereas it may not be essential for survival in COX of liver and other tissues.

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