Chemical Labeling Studies on Bovine Heart Mitochondrial Cytochrome *c* Oxidase Dispersed in Nonionic Detergents†

Lisa A. Estey, A. Jeannine Lincoln, and Lawrence J. Prochaska*

Department of Biochemistry, School of Medicine and College of Science and Mathematics, Wright State University, Dayton, Ohio 45435

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ABSTRACT: In order to investigate the structural interactions of nonionic detergents with bovine heart mitochondrial cytochrome *c* oxidase (COX), a series of hydrophilic chemical modification reagents were used to map regions on COX which are not shielded by dodecyl β-D-maltoside (DM), Triton X-100 (TX-100), and Tween 80 (TW-80). Low levels of incorporation of the chemical reagents [^{35}S]benzenediazoniumsulfonate (DABS) and *N*-succinimidyl [^{3}H]propionate (SP) into COX dispersed in TW-80 indicate that the bulky headgroup and hydrophobic moiety of this detergent effectively shield the enzyme from the aqueous environment. Subunits II and Va/Vb [nomenclature of Merle, P., & Kadenbach, B. (1982) *Eur. J. Biochem. 125*, 239–244] show an increased reactivity to [^{35}S]DABS and [^{3}H]SP in TW-80 and may reflect an increased exposure of these subunits to the aqueous phase in comparison to COX dispersed in TX-100 or DM. More [^{35}S]DABS is incorporated into COX in DM than TX-100-dispersed enzyme; DABS heavily labels subunits III, VIa, and VIb in DM. While COX in TX-100 is more reactive with [^{3}H]SP than DM-dispersed enzyme, there is no difference in the distribution of label (either DABS or SP) within the subunits of COX in DM or TX-100. Increased surface exposure of COX in TX-100 is indicated by an enhanced sensitivity of COX electron-transfer activity in enzyme chemically modified by the cross-linking reagent *N*-succinimidyl 3-[(4-azidophenyl)dithio]propionate (SADP) in TX-100 as compared to enzyme chemically cross-linked in the other detergents. These results suggest that the maltose headgroup of DM interacts with COX more strongly than the alkyl ether headgroup of TX-100, with TX-100–COX interactions stabilized by the short, bulky hydrophobic tail of the detergent. In the absence of favorable headgroup interactions, COX dispersed in TX-100 exhibits lower electron-transfer activity.

Cytochrome *c* oxidase, the terminal enzyme in the mitochondrial respiratory chain, catalyzes the oxidation of ferrocytochrome *c* and the reduction of molecular oxygen to water (Hatefi, 1985). In addition to its electron-transferring function, cytochrome *c* oxidase (COX)[1] facilitates vectorial proton translocation across the mitochondrial inner membrane (Wikstrom et al., 1981). COX from bovine heart mitochondria is well characterized; the oxidation–reduction centers, the three-dimensional structure of the enzyme, and the primary amino acid sequences and the surface topography of the subunits in the membrane are all known (Capaldi et al., 1987).

The effect of detergent environment upon COX activity has been well characterized (Rosevear et al., 1980; Vik et al., 1981; Robinson et al., 1985; Sinjorjo et al., 1987; Mahapatro & Robinson, 1990). Detergents affect COX electron-transfer activity at concentrations at or above the critical micelle concentration (CMC). Sinjorjo et al. (1987) have shown that micellar binding of detergents can have either positive or negative effects on steady-state electron transfer: at the CMC, dodecyl β-D-maltoside (DM) stimulates the electron-transfer rate, while cholate and Triton X-100 (TX-100) binding inhibits electron transfer [Tween-80 (TW-80) has no effect]. These observations suggest that the detergent headgroup is a major determinant in controlling electron-transfer activity in solution. In addition, variation of the hydrocarbon tails in the alkyl maltosides suggests that lauryl and myristyl hydrocarbons are slightly more effective than palmityl and stearyl tails in supporting electron-transfer activity (Robinson et al., 1985). As illustrated in the above examples, both headgroup and hydrophobic tail interactions play an important role in the stabilization of electron-transfer activity in COX in vitro.

Although detergent binding effects on both steady-state and transient electron-transfer kinetics are well characterized, the only structural data available to describe detergent interactions with COX are through studies of the effect of detergents on COX aggregation state (Robinson & Capaldi, 1977; Suarez et al., 1984; Robinson & Talbert, 1986). In an alternative approach, we have investigated the structural interactions of nonionic detergents with COX using chemical modification of protein functional groups. We have reacted COX dispersed in detergents commonly used for COX experimentation, TW-80, TX-100, and DM, with a battery of hydrophilic chemical modification reagents including benzenediazoniumsulfonate (DABS), *N*-succinimidyl propionate (SP), and the heterobifunctional chemical cross-linking reagent *N*-succinimidyl 3-[(4-azidophenyl)dithio]propionate (SADP). Since substantially different electron-transfer activities have been observed in these detergents, the chemical labeling patterns

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[1] Abbreviations: COX, cytochrome *c* oxidase; CMC, critical micelle concentration; DABS, benzenediazoniumsulfonate; DM, dodecyl β-D-maltoside; M_r, molecular weight; NAP-taurine, *N*-(4-azido-2-nitrophenyl)-2-aminoethanesulfonate; SADP, *N*-succinimidyl 3-[(4-azidophenyl)dithio]propionate; SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SP, *N*-succinimidyl propionate; TN, turnover number (moles of cytochrome *c* oxidized per mole of heme aa_3 per second); Tris, tris(hydroxymethyl)-aminomethane; TW-80, Tween-80; TX-100, Triton X-100.

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may discern structural differences in the interaction of the detergent headgroup and hydrophobic tails with the enzyme.

Experimental Procedures

Enzyme Preparations. Cytochrome *c* oxidase was isolated from mitochondria (Azzone et al., 1979) as described by Yonetani (1967). COX concentration was determined by using extinction coefficients of 24 mM^{-1} (Van Gelder, 1966) for heme aa_3 at 605 nm for reduced minus oxidized spectra and 33 mM^{-1} for reduced heme aa_3 at Δ605–630 nm (Briggs & Capaldi, 1977). The heme aa_3 concentration was determined by averaging the concentration obtained from both extinction coefficients. Protein was estimated by the method of Lowry et al. (1951).

Electron-Transfer Activity. After SADP treatment, aliquots of COX were incubated with 50 mM DM and activated in 4 mg/mL asolectin, 4.64 mM cholate, and 50 mM KH_2PO_4, pH 7.4, at a final concentration of 60 nM heme aa_3 for 16 h at 0 °C (DiBiase & Prochaska, 1985). Aliquots of activated enzyme (9.38 nM) were assayed polarographically at 25 °C in a buffer containing 0.6 mM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (recrystallized in ethanol/HCl) (Aldrich Chemical Co.), 35 μM cytochrome *c* (type III, Sigma Chemical Co.), 7 mM ascorbate, and 50 mM KH_2PO_4, pH 6.5, with 0.25 mM DM (Thompson & Ferguson-Miller, 1983).

Gel Filtration Chromatography. COX (20 nmol) was incubated with either 960 mol of TX-100, 610 mol of TW-80, or 780 mol of DM per mole of COX for 30 min at 0 °C and chromatographed over Sepharose 2B (1.75 × 30 cm) equilibrated with 90 mM NaCl, 20 mM KH_2PO_4, pH 8.0, and either 3.2 mM TX-100, 3.8 mM TW-80, or 1.96 mM DM. The A_{420nm} fractions were pooled, and the heme aa_3 concentration was determined. This was the starting material for all chemical modification experiments.

[^{35}S]DABS and [^{3}H]SP Labeling of COX. COX (0.6–2 μM aa_3) eluted from columns containing the various detergents was reacted with 25 μM [^{35}S]DABS (Amersham, 2 Ci/mmol) or 35 μM [^{3}H]SP [*N*-succinimidyl propionate-*2,3,t* (NEN–Du Pont, 53 mCi/mmol before dilution with nonradioactive SP)] for 15 min at 20 °C, and the reactions were quenched with 2 mM histidine (DABS) or 200 mM Tris-HCl, pH 8.5 (SP). The enzyme was collected by ultracentrifugation through 438 mM sucrose/10 mM Tris-HCl, pH 7.5, at 286000*g* overnight, dissociated at 37 °C for 30 min in 10% SDS, 5% β-mercaptoethanol, and 10 mM Tris–phosphate, pH 6.8, and run on 12.5% acrylamide–8 M urea cylindrical gels (Prochaska et al., 1981). After being stained with Coomassie Blue and destained, the gels were scanned at 550 nm with a Gilford 2600 spectrophotometer equipped with a linear transport. The gels were sliced into 1.2-mm sections with a gel slicer (Bio-Rad). The slices were digested with 50% H_2O_2 at 60 °C overnight, and 3.0 mL of Scintiverse Bio HP (Fisher) was added before counting on a Packard liquid scintillation counter (DiBiase & Prochaska, 1985).

SP was prepared from *N*-hydroxysuccinimide and propionic acid (both from Aldrich Chemical Co.) by using the dicyclohexylcarbodiimide method of ester synthesis of Anderson et al., (1964). Purity was assessed by thin-layer chromatography on silica gel plates developed with ethyl acetate/hexane (1/1 v/v) using [^{3}H]SP as the standard and was judged to be greater than 90%.

Chemical Cross-Linking of Cytochrome c Oxidase. COX (0.65–0.90 μM aa_3) from the gel filtration columns was reacted with varying concentrations of SADP (0–700 μM, Pierce Chemical Co.) in the dark for 15 min at room temperature and then irradiated with a Mineralite ultraviolet lamp [3 × 10^3 erg/cm^2·s] for 30 min at 0 °C to complete photoactivated cross-link formation; reactions were quenched with 50 mM Tris, pH 8.0. Aliquots of SADP-treated enzyme were pelleted by ultracentrifugation through 10 mM Tris-HCl, pH 7.4, 250 mM sucrose, and 2.32 mM cholate. COX was then dissociated in 100 mM Tris-HCl, pH 6.2, 8.0 M urea, and 5% SDS at 37 °C for 30 min. SDS-PAGE was performed by using either 16% acrylamide SDS–urea slab gels as described by Fuller et al. (1981) or 5–22% acrylamide–SDS-gradient slab gels as described by Kumar and Leffak (1986).

Results

The interaction of three nonionic detergents (DM, TW-80, and TX-100) which support variable degrees of electron-transfer activity in COX yet share physical properties such as large micellar size (>50 kDa) and low CMC (<0.2 mM) was studied. COX incubated and assayed in DM had high activity (TN = 600 s^{-1}), while TW-80-dispersed enzyme exhibited a rate of 240 s^{-1} in TW-80. COX incubated and assayed in TX-100 had low activity (28 s^{-1}), but the low activity was completely reversed by assaying in DM (600 s^{-1}). The ability of DM to effectively replace TX-100 on COX suggests that these two detergents share similar sites of binding (most likely hydrophobic domains on the enzyme).

Chemical Labeling of COX Incubated in Nonionic Detergents Using Hydrophilic Chemical Modification Reagents. In order to map sites of detergent interaction on COX, regions unshielded by detergent binding or protein–protein interactions were identified by using a series of water-soluble radioactive chemical modification reagents. DABS has been shown to react with histidine, lysine, tyrosine, and cysteine residues exclusively in the hydrophilic domains of membrane-spanning polypeptides, including subunit IV of COX (Malatesta et al., 1983). The enzyme was incubated in either excess DM, TX-100, or TW-80, chromatographed over Sepharose 2B to remove any aggregated enzyme and unbound detergent, and then labeled with 25 μM [^{35}S]DABS. Figure 1A shows the [^{35}S]DABS subunit labeling profile of COX when the enzyme was dispersed and reacted in DM. Figure 2A shows the quantitation of [^{35}S]DABS bound to each subunit when normalized for protein content on the SDS–PAGE gels in enzyme labeled in all three detergents. [^{35}S]DABS reacts with subunits III, VIIa–c, and VIII in all three detergents, while subunits Va and Vb are heavily labeled in TW-80 as shown by Prochaska et al. (1980). Also, VIb and VIa show increased labeling in both DM and TX-100. Subunit I appears to be labeled in Figure 1A; however, Prochaska et al. (1980) have shown that there is an impurity in the preparation of COX which has an electrophoretic mobility near subunit I that is highly labeled by [^{35}S]DABS. Subunit VIc is unreactive with [^{35}S]DABS in all detergents. Overall, more [^{35}S]DABS is bound to COX labeled in DM (0.2 mol of DABS/mol of COX) than enzyme dispersed and labeled in TX-100 (0.1 mol of DABS/mol of COX) and in TW-80 (0.07 mol of DABS/mol of COX).

The labeling data were also analyzed in terms of the percentage of reagent (or distribution) incorporated into each subunit. Table I shows the distribution of [^{35}S]DABS label into individual subunits. The distribution of [^{35}S]DABS into COX in DM and in TX-100 is similar, while the enzyme labeled in TW-80 exhibits a significant increase in the distribution of reagent into subunits II and Va. These results suggest that the surface exposure of the enzyme in DM more closely resembles the enzyme in TX-100, whereas different portions of the enzyme are exposed in TW-80. It should also be noted that the enzyme in DM is more highly labeled (when

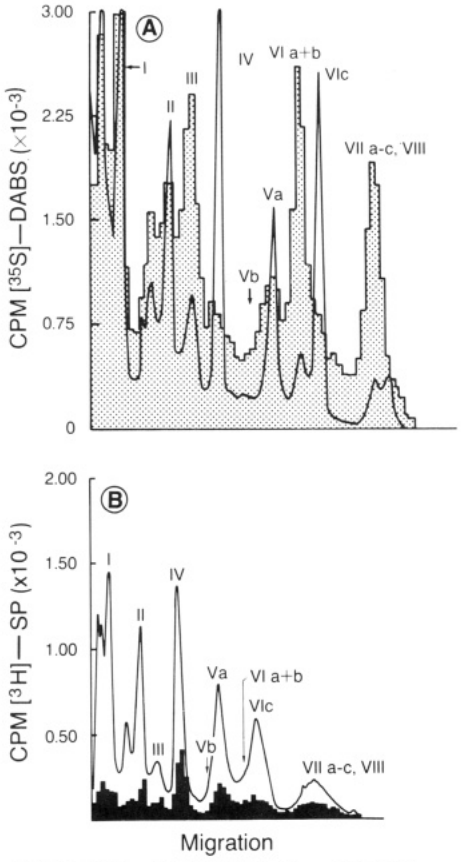

FIGURE 1: [^{35}S]DABS and [^{3}H]SP labeling of COX dispersed in DM. COX dispersed in DM was reacted with 25 μM [^{35}S]DABS (A) or 35 μM [^{3}H]SP (B), collected by ultracentrifugation, and run on 12.5% SDS–urea–acrylamide gels as described under Experimental Procedures. The densitometric trace of the COX subunits (indicated by the solid line) was plotted against the radioactivity [dotted (A) or solid (B) area] associated with each 1.2-mm slice of the acrylamide gel. The subunit nomenclature is that of Merle and Kadenbach (1982).

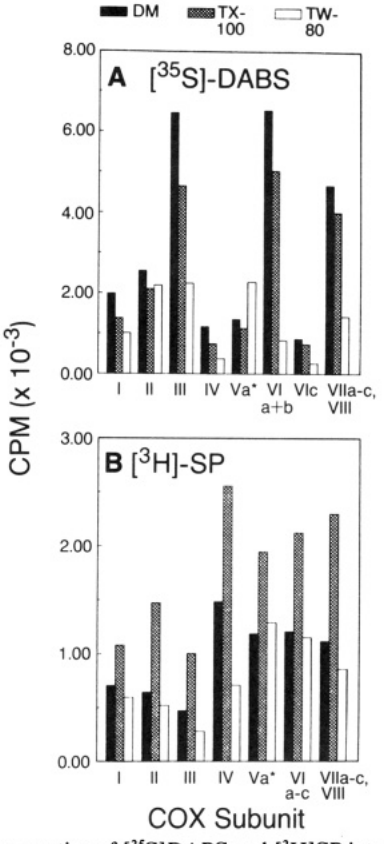

FIGURE 2: Incorporation of [^{35}S]DABS and [^{3}H]SP into the subunits of detergent-dispersed COX. This figure shows the amount of [^{35}S]DABS (A) or [^{3}H]SP (B) bound to each subunit of COX when modified in the different detergents. The data for DM was obtained by summing the radioactivity associated with each subunit in Figure 1 (with normalization for protein content on the gels) as well as for TX-100- and TW-80-dispersed COX. Va* represents the radioactivity incorporated into subunits Va and Vb.

Table I: Distribution of [^{35}S]DABS and [^{3}H]SP within Detergent-Dispersed COX[a] Label Incorporated (Percent of Total)

	[^{35}S]DABS			[^{3}H]SP		
subunit	DM	TX-100	TW-80	DM	TX-100	TW-80
I	7.8	7.0	9.5	9.2	8.7	11.0
II	10.0	10.6	*20.7*[b]	9.6	11.8	9.6
III	25.2	23.5	21.2	7.0	8.0	5.3
IV	4.6	3.9	3.5	21.9	20.5	*13.1*
Va, Vb	5.3	5.7	*21.4*	17.7	15.6	*23.8*
VIa, VIb	25.5	25.4	7.8	{18.0[c]	17.0	21.4}
VIc	3.4	3.7	2.5			
VIIa–c, VIII	18.2	20.2	*13.4*	16.6	18.4	15.8

[a] The data in any column demonstrate the percent distribution of [^{35}S]DABS and [^{3}H]SP within the subunits of COX when the enzyme was modified in DM, TX-100, and TX-80. Presented is the amount of radioactivity (in cpm from Figure 2) bound to each subunit expressed as a percentage of the total cpm incorporated into COX. The sum of each column is 100% by definition. [b] Data in *italics* indicate values that are significantly different in TW-80 as compared to DM- or TX-100-dispersed COX. [c] The brace indicates values for subunits VIa–c, which are combined in the [^{3}H]SP labeling data.

normalized for amount of protein) than in other detergents, suggesting that more [^{35}S]DABS reactive sites are exposed in DM.

The surface topography of COX in the different detergents was also mapped by using the lysine-directed reagent [^{3}H]SP. [^{3}H]SP has limited solubility in water (<10 mM) and most likely will react with lysine residues at or near the headgroups of the detergents. The [^{3}H]SP subunit labeling profile of COX dispersed in DM is presented in Figure 1B, and Figure 2B quantitates the amount of [^{3}H]SP bound to the subunits of COX dispersed in the three detergents. Subunits IV and Va are highly labeled in all detergents (Figure 2B). Also, subunits I and VIc, while being unreactive with [^{35}S]DABS (Figure 2A), are significantly labeled by [^{3}H]SP in all detergents. The low level of [^{3}H]SP labeling in the highly exposed subunit III is probably due to its low lysine content (Buse et al., 1985). The enzyme in TX-100 is more reactive with [^{3}H]SP than enzyme dispersed in DM or TW-80 (Figure 2B). The distribution of [^{3}H]SP labeling of COX in TW-80 is different (less reactivity of IV, more labeling of subunit Va) (Table I), and there is less total reagent incorporated into COX in TW-80 (0.49 mol of [^{3}H]SP/mol of COX than COX dispersed in the other detergents (0.62 and 1.14 mol of [^{3}H]SP/mol of COX in DM and TX-100, respectively). A quantitative comparison of [^{3}H]SP and [^{35}S]DABS labeling has limitations due to the differences in the total amount of reagent bound (moles of reagent bound per mole of COX); however, comparisons of the data for one reagent in the three detergents can be analyzed. Within these restrictions, it still appears that the TX-100-dispersed enzyme contains several lysine residues which are not shielded by detergent yet are inaccessible to negatively charged modification reagents such as [^{35}S]DABS.

Inhibition of Electron-Transfer Activity of COX Induced by a Chemical Cross-Linking Reagent in Different Detergents. One possible explanation for the differences in COX electron-transfer activity is that the nonionic detergents bind to different domains on the enzyme, thus changing the environment or conformation of the enzyme during the steady-state

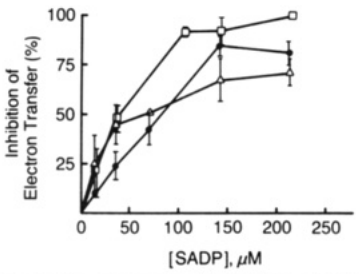

FIGURE 3: Concentration-dependent inhibition of electron-transfer activity of detergent-dispersed COX induced by SADP. COX dispersed in DM (●), TX-100 (□), or TW-80 (△) was reacted with 0–220 μM SADP as described under Experimental Procedures. Aliquots of each reaction were activated in 4 mg/mL asolectin, 0.2% cholate, and 50 mM KH_2PO_4, pH 7.4. Polarographic electron-transfer assays were performed as described in Thompson and Ferguson-Miller (1983). Inhibition of electron-transfer activity was reported as a percentage of control (UV-irradiated sample); control turnover numbers (in s^{-1}) were 279, 226, and 197 for COX dispersed in DM, TX-100, and TW-80, respectively.

assay. This idea was tested further by reacting the enzyme in each detergent with various concentrations of a heterobifunctional chemical cross-linking reagent, SADP, and then measuring the electron-transfer activity in DM (Figure 3), a detergent which allows maximum electron-transfer activity. Complete inhibition of electron-transfer activity induced by SADP was only observed when COX was reacted in TX-100 (Figure 3), while when reacted in DM and TW-80 (at concentrations up to 0.75 mM for COX dispersed in TW-80) COX failed to exhibit 100% inhibition of activity. Overall, less SADP was required to cause 50% inhibition of electron-transfer activity when the enzyme was incubated in TX-100 (35 μM) than enzyme in TW-80 (59 μM) and DM (71 μM) (Figure 3). These results suggest that enzyme incubated in TX-100 exhibits a different reactivity with SADP than the enzyme in TW-80 or DM and, presumably, the sites that lead to inhibition of electron-transfer activity are more exposed to the cross-linking reagent in TX-100 than in DM or TW-80. Alternatively, SADP cross-linking of COX in TX-100 locks the enzyme into a conformation with low electron-transfer activity as suggested by Mahapatro and Robinson (1990).

SDS–PAGE of SADP-Modified COX Reacted in Nonionic Detergents. Aliquots of the detergent-dispersed enzyme were modified with SADP and analyzed for formation of intersubunit cross-links by SDS-PAGE (Figure 4)to investigate further the reactivity of detergent-dispersed COX with SADP. Two polyacrylamide gel systems were used: A 16% acrylamide–6 M urea gel was used to maximally separate cross-linked subunit pairs in the 5–40-kDa range (Figure 4A), while a 5–22% acrylamide gradient gel with 8 M urea (Figure 4B) was used for separation of cross-linked subunits between 25 and 300 kDa. Figure 4A,B shows that the enzyme dispersed in TX-100 exhibits the formation of numerous intersubunit cross-links (indicated by the stars on the left margin of the figure and dots to the right of the lanes within the gel) when reacted with SADP and that the concentration dependence of their formation correlates with the degree of inhibition of electron-transfer activity (Figure 3). More intersubunit cross-links were formed at concentrations of SADP which lead to increased levels of electron-transfer inhibition (compare lanes 2 and 6 in any detergent in Figure 4A). Intersubunit cross-links migrating between subunits I and II (Figure 4A) and at molecular weights greater than that of subunit I [including species that were unable to enter the gel (M_r >60K)] were observed. These larger intersubunit cross-links (Figure 4B) were further analyzed by gradient gel SDS–PAGE, and at the higher concentrations of SADP, some cross-links were unable to enter the gel (M_r >324K).

The formation of cross-linked subunit pairs required less SADP for enzyme reacted in TX-100 than in TW-80 or DM (Figure 4A,B). At any one concentration of SADP, less cross-linked subunits were observed in enzyme modified in DM and TW-80 as compared to TX-100-dispersed COX. The intersubunit cross-links formed in all three detergents appear to have similar molecular weights. Two intersubunit cross-links were identified by the use of purified antibodies to the subunits of COX; one at a molecular weight of 38K and another at 33K, both containing subunits II + Va (data not shown). These two intersubunit cross-links are apparently formed in all detergents. No unique intersubunit cross-links were detected in any detergent. The formation of intersubunit cross-links in the detergents as assayed by SDS–PAGE supports the idea that the enzyme in TX-100 is more reactive with

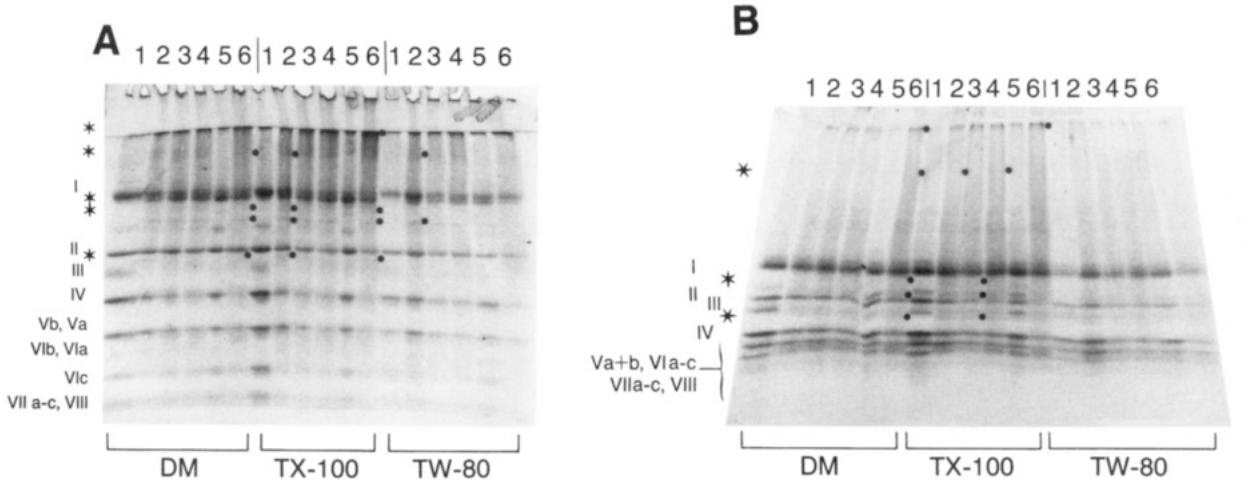

FIGURE 4: SDS–PAGE of COX reacted with SADP in different nonionic detergents. Chemical cross-linking of COX with SADP was performed as described under Experimental Procedures, and aliquots of the enzyme were subjected to SDS–PAGE through either 16% acrylamide (panel A) or 5–22% acrylamide gradient gels (panel B). In panels A and B, lanes 1–6 contain DM-, TX-100-, or TW-80-dispersed COX reacted with the following concentrations of SADP: (1) 0, (2) 18.0, (3) 35.5, (4) 53.2, and (5) 35.5 μM (no UV irradiation); (6) 71.0 μM. The stars on the left indicate the migration of intersubunit cross-links induced by SADP. The dots within the gel (on the right side of each lane) show intersubunit cross-links created by SADP in the different detergents. In gel A, similar intersubunit cross-links occur in lane 6 of COX reacted in DM and in lane 2 of COX reacted in TX-100 (see dots). In gel B, new bands (intersubunit cross-links) occur in lane 6 for enzyme reacted in DM and also in lane 3 for COX reacted in TX-100 (see dots). In gel A, the decreased staining intensity of subunit III (lanes 2–4, 6) is due to formation of intersubunit cross-links involving this subunit.

chemical reagents than COX dispersed in TW-80 or DM.

Discussion

Markedly different rates of electron transfer of COX have been observed in the detergents used in this study, ranging from values of 10 s^{-1} in TX-100 (Robinson et al., 1985; Sinjorgo et al., 1987) to 600 s^{-1} in DM (Thompson & Ferguson-Miller, 1983; Hill & Robinson, 1986). The differences in electron-transfer rates in the detergents could be explained by many possible mechanisms. First, the detergents may have distinct modes of binding to COX influenced by the chemical differences in the headgroup and hydrophobic tail of the detergent. Second, the binding of the detergents may induce conformational changes in the enzyme which result in different rates of electron transfer. Third, the detergents may change the oligomeric state of COX by disrupting protein–protein interactions, creating different electron-transfer activities in each detergent.

In this work, we used a series of hydrophilic chemical modification reagents to identify differences in DM, TX-100, and TW-80 interactions with COX. Using this approach, it is expected that detergents with bulky headgroups (i.e., TW-80), or detergents which interact strongly with the enzyme, should exhibit lower reactivity with the hydrophilic reagents. Flexible detergent association or smaller headgroups will lead to an increase in reagent incorporation. The three hydrophilic reagents used to label COX in the detergents were DABS, SP, and SADP. DABS, a negatively charged reagent, was used because it has a broad amino acid specificity (Lys, His, Tyr, and Cys) and has been used in surface topography studies on COX (Eytan et al., 1975; Ludwig et al., 1979; Prochaska et al., 1980; Malatesta et al., 1983). SADP, a heterobifunctional chemical cross-linking reagent, was used because it has limited specificity (α- and ϵ-amino groups) on one end of the molecule, while the other end (a nitrene after photoactivation) has *relatively* nonspecific reactivity with many amino acids (Richards & Brunner, 1980). SP represents the amino group directed end of SADP and is radioactively labeled, thus allowing identification of possible SADP binding sites. Both SP and SADP have limited aqueous solubility; however, their specificities for lysine residues allow them to map hydrophilic areas of COX (Malatesta et al., 1983; Zhang et al., 1988).

Our labeling results show that the enzyme dispersed in TW-80 is much less reactive to *all* reagents than COX in DM and TX-100. In addition, to decreased reactivity with all of the reagents employed, the enzyme dispersed in TW-80 shows a different distribution of DABS and SP labeling as well as decreased sensitivity to SADP-induced inhibition of electron transfer. This low reactivity of the reagents with COX in TW-80 suggests that the steric bulkiness of the headgroup of TW-80 limits the accessibility of the reagents to COX by covering putative reactive sites. Another distinct possibility is that TW-80 is unable to disperse large COX aggregates and limits accessibility of the modification reagents.

COX dispersed in DM is more reactive to the reagents than the enzyme in TW-80. COX is more reactive with DABS in DM than COX in TX-100, but the enzyme in DM is *less* reactive with SP and SADP than COX dispersed in TX-100. The increase in DABS incorporation into enzyme modified in DM is primarily due to labeling in subunits III, VIa and VIb, presumably because of high histidine content in III and VIa (Buse et al., 1985). In our view, the SP labeling results suggest that the maltose headgroup of DM shields sites on COX similar to TX-100, but the strength of the interaction of the headgroup of DM with the hydrophilic domains of COX is greater than in TX-100–COX interactions. DM, with its highly defined maltose headgroup, is likely to be more tightly associated with COX and have less flexibility once bound to COX than TX-100, although the absolute amount of DM and TX-100 bound to the enzyme appears to be similar [200 mol of DM and 180 mol of TX-100 per mole of COX, respectively (Suarez et al., 1984; Robinson & Capaldi, 1977)]. The maltose headgroup and the C12 alkyl side chain of DM satisfy both hydrophilic and hydrophobic requirements for maximum COX electron-transfer activity (Rosevear et al., 1980; Robinson et al., 1985).

COX dispersed in TX-100 is more reactive with the hydrophilic reagents than TW-80-dispersed COX. As stated previously, the enzyme dispersed in TX-100 is less reactive with DABS and more reactive with SP and SADP than enzyme incubated in DM. Our SP labeling results suggest that COX in TX-100 has more exposed lysine-reactive groups and a more flexible association with hydrophilic COX domains than does DM. The binding of TX-100 to integral membrane proteins occurs through the hydrophobic portion of the detergent with little or no contributions of the headgroup (Helenius & Simons, 1975). The flexible alkyl ether headgroup of TX-100 may not associate as tightly with COX as DM, leading to increased reactivity with SP and SADP when dispersed in TX-100 than enzyme in DM. Alternatively, DM and TX-100 could have similar sites of interaction with COX, but different conformational states may exist in TX-100 and DM as first suggested by Robinson et al. (1985). Our SADP cross-linking data support a conformational modification of COX in TX-100 but cannot unequivocally distinguish between the two possibilities. Recent evidence suggests that COX in TX-100 is unable to attain the pulsed conformational state (Mahapatro & Robinson, 1990) and is responsible for the low steady-state electron-transfer rates observed in this detergent.

Our data also exemplify the limitations of chemical modification reagents in the study of membrane-intercalated proteins. In our view, SP and SADP may be reagents that derivatize lysine residues at the level of the detergent headgroups due to their limited solubility in water and their neutral charge. It is unlikely that SP is reacting from within the detergent micelle due to the low level of SP label incorporated into subunit I, which is the most hydrophobic subunit as determined by both amino acid composition (Buse et al., 1985) and azidophospholipid labeling (Prochaska et al., 1980). The SP labeling that we observed in subunit VIc [as well as in NAP-taurine (Prochaska et al., 1980) and in the water-soluble benzaldehyde (McMillen et al., 1986) labeling studies of COX] gives some support to this idea, since surface topographical studies suggest a transmembrane orientation for this subunit with six lysine residues on the cytoplasmic side of the membrane (Zhang et al., 1988). DABS does not label subunit VIc in nondenaturing detergents (Figure 1; Ludwig et al., 1979; Prochaska et al., 1980), suggesting that at least for subunit VIc labeling, the electrostatic repulsion of DABS, possibly at the level of the detergent headgroup, may occur. Subunit IV reactivity with DABS may also be explained by this mechanism.

One limitation in our experiments is that COX exists in different oligomeric states in the nonionic detergents, perhaps causing protein–protein interactions to affect our interpretations of COX–detergent interactions. COX is a monodisperse monomer in DM [Suarez et al., 1984; although see Bolli et al. (1985)]. The enzyme also is a monodisperse monomer in high TX-100 (5 mg/mg of protein), but at lower stoichiometries of TX-100 to COX, a heterogeneity of aggregation states occurs with a polydisperse mixture of momomers and dimers

predominating (Robinson & Capaldi, 1977; Robinson & Talbert, 1986). In TW-80, COX is a polydisperse mixture of dimers or higher aggregated forms (Robinson & Capaldi, 1977). In our TX-100 experiments (3 mg of TX-100/mg of protein), it is likely that COX is a monomer (<10% dimer) as shown by sedimentation velocity measurements (L. A. Estey, and L. J. Prochaska, unpublished results). The oligomeric state of COX is unlikely to have a significant effect on our results for two reasons. First, our results show that the enzyme is more reactive with SP and SADP in TX-100, where the enzyme is a mixture of monomers and dimers, than in DM, where the enzyme is most likely in the monomeric form. Monomers would be expected to react with the reagents to a greater extent than dimers due to the exposure of monomer–monomer contact sites. Second, the reagent concentrations employed in our chemical modification studies were much less than saturating (0.5–1.14 mol of SP bound/mol of COX as compared to 85 mol of lysine/mol of COX), and therefore less likely to reflect differences due to the oligomeric state. Similar reasoning also makes it unlikely that the observed differences in labeling of COX in DM and TX-100 are due to conformational rearrangements induced indirectly by changing the oligomeric state of the enzyme. This indirect mechanism cannot be eliminated for our labeling results in COX dispersed in TW-80, although the enzyme in TW-80 is much less reactive than COX in the other detergents.

Our chemical labeling results allow us to make the following conclusions about the interaction of these three detergents with COX. DM, with its defined maltose headgroup and 12-carbon hydrophobic group (Rosevear et al., 1980; Robinson et al., 1985), is the most suitable detergent for assaying COX electron-transfer activity because it has both a highly favorable hydrophobic group to interact with the membrane-intercalated domains of COX and a polar headgroup that can strongly interact with the hydrophilic domains of COX. The headgroup of DM binds to similar sites as TX-100, but shields the enzyme more effectively from the hydrophilic chemical reagents than TX-100, suggesting a tighter headgroup–protein interaction in DM. TX-100 interacts with COX through its hydrophobic portion and has limited headgroup interaction with the hydrophilic domains of COX as shown by increased reactivity with the hydrophilic reagents. The decreased headgroup interaction of TX-100 could explain the low electron-transfer activity of COX in this detergent. Alternatively, the strong hydrophobic interactions of TX-100 with COX may lock COX in an altered, low-activity conformational state (the resting form of the enzyme) (Mahapatro & Robinson, 1990) that increases the reactivity of COX with SADP. Finally, the hydrophilic and hydrophobic interactions of TW-80 with COX are strong enough to satisfy the requirement for COX electron-transfer activity, although the headgroup of this detergent is bulky and shields the enzyme from chemical modification. TW-80 also lacks the hydrophobic dispersing power of DM to disrupt aggregated COX into highly active monomers, yet these aggregates are able to maintain electron-transfer activity.

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Registry No. COX, 9001-16-5; DM, 69227-93-6; TW-80, 9005-65-6; TX-100, 9002-93-1.

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Kinetics of Nucleotide Transport in Rat Heart Mitochondria Studied by a Rapid Filtration Technique†

Gérard Brandolin,* Isabelle Marty, and Pierre V. Vignais
LBIO/Laboratoire de Biochimie, Département de Recherche Fondamentale, Centre d'Etudes Nucléaires, 85X, 38041 Grenoble Cedex, France

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ABSTRACT: A rapid filtration technique has been used to measure at room temperature the kinetics of ADP and ATP transport in rat heart mitochondria in the millisecond time range. Transport was stopped by cessation of the nucleotide supply, without the use of a transport inhibitor, thus avoiding any quenching delay. The mitochondria were preincubated for 30 s either in isotonic KCl containing succinate, $MgCl_2$, and P_i (medium P) or in isotonic KCl supplemented only with EDTA and Tris (medium K); they were referred to as energized and resting mitochondria, respectively. The kinetics of [^{14}C]ADP transport in energized mitochondria were apparently monophasic. The plateau value for [^{14}C]ADP uptake reached 4-5 nmol of nucleotide·(mg of protein)$^{-1}$. V_{max} values for [^{14}C]ADP transport of 400-450 nmol exchanged·min^{-1}·(mg of protein)$^{-1}$ with K_m values of the order of 13-15 μM were calculated, consistent with rates of phosphorylation in the presence of succinate of 320-400 nmol of ATP formed·min^{-1}·(mg of protein)$^{-1}$. The rate of transport of [^{14}C]ATP in energized mitochondria was 5-10 times lower than that of [^{14}C]ADP. Upon uncoupling, the rate of [^{14}C]ATP uptake was enhanced, and that of [^{14}C]ADP uptake was decreased. However, the two rates did not equalize, indicating that transport was not exclusively electrogenic. Transport of [^{14}C]ADP and [^{14}C]ATP by resting mitochondria followed biphasic kinetics. These consisted of a rapid nucleotide uptake of about 350 nmol·min^{-1}·(mg of protein)$^{-1}$, lasting for about 1 s, up to 1.0-1.2 nmol of [^{14}C]nucleotide taken up·(mg of protein)$^{-1}$, followed by a slow phase leading to a plateau value of 4-5 nmol of [^{14}C]nucleotide·(mg of protein)$^{-1}$, which was attained in 1 min. Depletion of nucleotides in resting mitochondria resulted in a greater decrease in the extent of the slow phase than of the rapid one. In addition, about half of the nucleotides taken up at the end of the rapid phase were not discharged into the medium upon addition of carboxyatractyloside. This suggested that matricial nucleotides are compartmentalized in two pools which are exchangeable at different rates with external nucleotides.

The kinetic data so far reported for the transport of adenine nucleotides by the mitochondrial ADP/ATP carrier have been obtained from "inhibitor-stop" experiments, using the specific inhibitors atractyloside (ATR)[1] or carboxyatractyloside (CATR) when transport was assayed with mitochondria (Vignais et al, 1985; Klingenberg, 1985) and bongkrekic acid (BA) in the case of inverted submitochondrial particles (Lauquin et al., 1977). The temperature was routinely lowered to 0-5 °C to slow down transport. A rapid filtration method has been recently developed (Dupont, 1984) which allows measurement of transport at room temperature in the millisecond time range without the use of an inhibitor to stop the reaction. This technique is based on perfusion of immobilized particles with a solution of labeled substrate and termination of transport by cessation of the perfusion. It has been successfully used to determine the initial rates of Ca^{2+} binding and transport in sarcoplasmic reticulum (Dupont, 1984) and of P_i transport in liver mitochondria (Ligeti et al., 1985). In the present work, we have applied the rapid filtration method to the study of the rapid kinetics of ADP/ATP transport in rat heart mitochondria at room temperature under different metabolic conditions. Some of the results suggest the existence of microcompartmentation of mitochondrial adenine nucleotides.

Experimental Procedures

Materials. Cellulose nitrate filters (0.8 μm, AAWP) were from Millipore. [^{14}C]Nucleotides and [^{3}H]dextran were from Amersham. Tritiated ATR and BA were synthesized as previously described [see Vignais et al. (1985)]. All other reagents were of the highest purity commercially available.

Methods. Rat heart mitochondria were prepared according to the method of Mela and Steitz (1979). Their protein content was determined by the biuret method (Gornall et al., 1949).

Prior to the transport step, the rat heart mitochondria were incubated at room temperature at a concentration of 1 mg/mL, either for 30 s in 125 mM KCl, 10 mM NaP_i, 5 mM $MgCl_2$, and 10 mM sodium succinate, pH 7.3 (medium P),

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[1] Abbreviations: ATR, atractyloside; CATR, carboxyatractyloside; BA, bongkrekic acid; Tris, tris(hydroxymethyl)aminomethane; MOPS, 4-morpholinepropanesulfonic acid; TCA, trichloroacetic acid; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; POPOP, 1,4-bis(5-phenyl-2-oxazolyl)benzene; PPO, 2,5-diphenyloxazole.

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