Covalent Modification of Hydrophilic and Hydrophobic Domains of Yeast Cytochrome *c* Oxidase with Fluorescent Azides†

Michael Edward Dockter* and Theresa Koseki

ABSTRACT: Two photolabile nitrene precursors, 3-azido-2,7-naphthalenedisulfonate and 5-azido-1-naphthalenesulfonate, have been synthesized, characterized, and used to determine the hydrophilic and hydrophobic domains of yeast cytochrome *c* oxidase. The highly charged 3-azido-2,7-naphthalenedisulfonate can be used to label those portions of the oxidase which are in contact with the aqueous solvent [Dockter, M. E. (1979) *J. Biol. Chem. 254*, 2161]. The more hydrophobic 5-azido-1-naphthalenesulfonate is an amphipathic probe and is expected to insert into hydrophobic regions of phospholipids or detergent micelles. Thus, this label would interact with the hydrophobic domains of purified yeast cytochrome *c* oxidase solubilized in Tween 80 detergent. When activated by light, these two aryl azides produce highly reactive nitrenes which are capable of covalent modification of regions of the macromolecular complexes with which they are in proximity to. The products of the photolysis reactions are highly fluorescent. The analysis of the fluorescence spectral characteristics of the photolysis products has allowed us to confirm the prediction that the 3-azido-2,7-naphthalenedisulfonate does indeed react at hydrophilic domains and that the 5-azido-1-naphthalenesulfonate reacts at hydrophobic domains of the detergent-solubilized oxidase. When purified, detergent-solubilized yeast cytochrome *c* oxidase is labeled with 3-azido-2,7-naphthalenedisulfonate, all polypeptide subunits with the exception of subunit V incorporate fluorescent label, suggesting that these subunits are situated, at least in part, exposed to the hydrophilic aqueous media. By contrast, when oxidase is labeled with the hydrophobic 5-azido-1-naphthalenesulfonate, the incorporated label is found predominantly in subunits I, II, III, and VII, suggesting that these subunits are localized in the more hydrophobic domains of the oxidase. The general utility of these probes and the relationship of these results to previous studies on yeast cytochrome *c* oxidase structure are discussed.

Cytochrome *c* oxidase is the terminal respiratory chain complex of the inner mitochondrial membrane. As such, it is responsible for the transfer of electrons from reduced cytochrome *c* on the cytoplasmic side of the inner mitochondrial membrane to molecular oxygen on the matrix side of this membrane. Much of the work on the structure and function of cytochrome *c* oxidase has focused on cell systems which are amenable to growth in culture and whose genetics are open to manipulation, like yeast and *Neurospora*. Isolated from baker's yeast cells in a highly purified form, cytochrome *c* oxidase has been characterized as containing seven distinct polypeptide chains varying in molecular weight from 56 000 down to a minimum of about 7000 (Schatz & Mason, 1974). The three subunits of highest molecular weight have been shown to be synthesized in the mitochondria and coded for by mitochondrial DNA (Bonitz et al., 1980; Coruzzi & Tzagoloff, 1979; Fox, 1979; Thalenfeld & Tzagoloff, 1980). The remaining four lower molecular weight polypeptides are coded for by nuclear DNA and synthesized on cytoplasmic ribosomes (Mason & Schatz, 1973; Rubin & Tzagoloff, 1973). Only subunit VI of the nuclearly coded yeast oxidase subunits has been sequenced (Gregor & Tsugita, 1982). In addition to the seven polypeptide chains, yeast cytochrome *c* oxidase is known to contain four prosthetic groups, namely, two hemes designated heme *a* and a_3 and two coppers.

In order to understand how the cytochrome *c* oxidase functions as a respiratory chain component as well as to understand how two component parts of cytochrome *c* oxidase are assembled in the inner membrane of the mitochondria, it is essential that we understand the topographical arrangements of the component parts in the functional complex. To this end, a number of studies have been undertaken with yeast cytochrome *c* oxidase to determine which of the polypeptide subunits are localized in the hydrophilic and hydrophobic domains of the complex. The labeling studies of the hydrophilic domains of yeast cytochrome *c* oxidase have been carried out with "surface labels" which, in general, interact specifically with reactive nucleophiles present on exposed subunits of the oxidase. These labels have included the widely used lactoperoxidase–iodination technique and *p*-benzenediazoniumsulfonate labeling (Eytan & Schatz, 1975). Labeling of the hydrophobic domains of yeast cytochrome *c* oxidase has been carried out with what are generally thought of to be "nonspecific labels", namely, the highly reactive aryl nitrenes produced by flash photolysis of hydrophobic aryl azides (Cerletti & Schatz, 1979; Gutweniger et al., 1981). Criticism of the former type of surface-labeling reagents centers around the obvious possibility that polypeptides of complexes may be exposed on the surface of a complex but may lack the appropriate nucleophile to react. Criticisms of the use of hydrophobic aryl azides as probes of the nonaqueous regions of membranes and membrane proteins have focused on the possibility that the reaction sites of the nitrene intermediates are the hydrophilic rather than the hydrophobic domains of membrane proteins (Bayley & Knowles, 1978a,b).

This laboratory has undertaken the design of a series of hydrophilic and hydrophobic probes useful in determining membrane topography. These probes are based on the previously described aryl azides which have the potential of nonspecific reaction (i.e., not requiring a specific nucleophile to be present) and thus can form covalent bonds through insertion reactions through carbon–hydrogen, nitrogen–hydrogen, or oxygen–hydrogen bonds (Smith, 1970). In addition,

† From the Department of Biochemistry, St. Jude Children's Research Hospital, and the Department of Biochemistry, The University of Tennessee Center for the Health Sciences, Memphis, Tennessee 38101. *Received November 24, 1982.* This work was supported by Grant AM-21974 and CORE Grant CA-21765 from the National Institutes of Health, American Cancer Society Institutional Grant IN-99, and ALSAC.

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the central structural feature of these aryl azides is that of naphthalenesulfonates which have well-documented, useful fluorescent characteristics (Hudson & Weber, 1973). This serves two useful purposes; namely, first, it allows the labeled polypeptides to be identified by their acquired fluorescence rather than relying on the more laborious and hazardous techniques of radiolabeling, and second, it allows us to critically analyze the microenvironment of the probe sites after photolysis, in situ, through standard fluorescence spectroscopy techniques. This second point allows the investigator to directly address the question of the actual environment of the photolysis products of hydrophilic and hydrophobic azides after reaction has taken place.

In the following experiments, we will show that the previously synthesized hydrophilic probe 3-azido-2,7-naphthalenedisulfonate (Moreland & Dockter, 1980; Dockter, 1979) is capable of labeling six of the seven subunits of purified, detergent-solubilized yeast cytochrome *c* oxidase, therefore suggesting that these subunits of the complex are, at least in part, exposed to the hydrophilic aqueous media. A second probe, 5-azido-1-naphthalenesulfonate, is amphipathic and can be shown to interact strongly with the hydrophobic domains of the detergent micelles in which the purified oxidase is solubilized. Only subunits I, II, III, and VII of yeast cytochrome *c* oxidase are apparently in contact with these hydrophobic reaction sites of this probe upon photolysis. The results are discussed with respect to previous topographical studies of yeast cytochrome *c* oxidase using more conventional labeling techniques.

Materials and Methods

Cytochrome c Oxidase. Cytochrome *c* oxidase was prepared from commercially grown bakers' yeast (Federal brand) by using a previously published procedure (Dockter et al., 1978). It contained between 12 and 15 nmol of heme *a*/mg of protein and was capable of oxidizing 150–170 μmol of ferrocytochrome *c* min^{-1} (mg of purified oxidase)$^{-1}$ at 25 °C in standard spectral assay conditions. The purified oxidase was stored at −80 °C in small aliquots in a buffer containing 20 mM sodium phosphate buffer, pH 7.4, and containing 1% Tween 80 detergent. The frozen oxidase samples were at a concentration of 10–20 mg/mL, and repetitive freezing and thawing of the sample were avoided as it resulted in some loss of oxidase activity.

Reagents. The 3-Amino-2,7-naphthalenedisulfonate and the 5-amino-1-naphthalenesulfonate were purchased as technical grade from Eastman Kodak and were recrystallized before use from HCl. ANMS[1] was synthesized according to the published procedure involving diazotization and subsequent immediate azide formation from 5-amino-1-naphthalene sulfonate (Moreland & Dockter, 1980). However, this previously published procedure, when used to synthesized ANDS, often resulted in incomplete conversion of the diazonium intermediate to the aryl azide, dependent on temperature and acid concentration of the reaction mixture. Thus, the ANDS was synthesized according to a slight modification of these published procedures which involves isolation of the intermediate diazonium salt. Working under a red safelight, 4.0 g (13.2 mmol) of recrystallized 3-amino-2,7-naphthalenedisulfonic acid was dissolved in 16 mL of sulfuric acid and heated to 55 °C to give a clear light green mixture. The sample was cooled to 0 °C, and a solution of 3.0 g (43.5 mmol) of sodium nitrite in 16 mL of water was added dropwise over a period of about 5 min. Immediately, 160 mL of ice water was added, which produced a yellow precipitate. The precipitate was allowed to dissolve by heating the sample to 40 °C and the orange mixture filtered. Upon cooling to 0 °C, the precipitate reappeared and was collected by filtration and washed at 0 °C with water, acetone, and finally hexane. This diazonium intermediate was dried in vacuo and 1 g (1.35 mmol) subsequently dissolved in 12 mL of water containing 0.6 g (9.23 mmol) of sodium azide. The reaction mixture was stirred at room temperature for 3 h to assure the complete conversion of the diazonium salt to the aryl azide. One hundred seventy-five milliliters of acetone was added to precipitate the product which was filtered and washed with acetone and air-dried. Due to the evolution of toxic HN_3 and NO_2 gas during the synthesis, the use of a well-ventilated area was essential. Yields were approximately 35% and 15% for ANDS and ANMS, respectively. Purity of the labels was determined by high-performance liquid chromatography and spectral analysis as has been described. These compounds have been stored at −20 °C in the dark for over 2 years without degradation. As they are potentially explosive, they should be handled with the appropriate caution. Tritiated ANDS was prepared by submitting recrystallized 3-amino-2,7-naphthalenedisulfonate to Amersham Corp. for catalytic tritium-exchange reaction in aqueous media (method TR.1). The resultant tritiated ANDS precursor was diluted 5-fold with cold 3-amino-2,7-naphthalenedisulfonate and recrystallized by acidification. Subsequently, this radiolabeled precursor was used to synthesize tritiated ANDS which had a specific activity of 32.3 mCi/mmol. Tritiated ANDS was stable when stored in dark at −20 °C for over 6 months. The nonionic detergent Tween 80 and horse heart cytochrome *c* type II were purchased from Sigma Chemical Co. and used without further purification. Lactoperoxidase was the kind gift of Dr. M. Morrison, and ^{125}I was purchased from Schwarz/Mann. All other chemicals were of analytical grade.

Photochemical Labeling. Solutions of 2 mg/mL purified cytochrome *c* oxidase dissolved in 20 mM NaP_i buffer, pH 7.4, containing 1% Tween 80 detergent were prepared. In the dark, a concentrated solution of ANDS or ANMS in distilled water was added such that the final concentration of the label in the solution was 1.0 mM. The resulting mixture was placed in a small watch glass such that the depth of the solution never exceeded 3 mm, and the entire solution was illuminated with a hand-held, high-intensity ultraviolet light with a maximum intensity at 366 nm (Ultraviolet Products UV L56). The irradiation time was routinely for 5 min at a distance of 5 cm at 30 °C. The time course of the photolysis could be estimated from the fluorescence produced (Dockter, 1979). The sample volumes were normally maintained at less than 0.5 mL. After irradiation, the labeled oxidase was removed from the bulk of the unbound label and Tween 80 detergent by passage through a Sephadex G-100 10-cm column containing 10 mM NaP_i, pH 7.4, buffer and 2% sodium cholate. The oxidase was collected and applied directly to sodium dodecyl sulfate/polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol by using either a 10–17% exponential gradient of acrylamide with a 5% stacking gel (Douglas et al., 1979) or a 10–20% step gel system (Cabral & Schatz, 1979). The bulk of the Tween 80 detergent was replaced with cholate before gel electrophoresis because the Tween 80 caused streaking of the lower molecular weight oxidase polypeptides on the gels. After completion of the gel runs, the incorporation

[1] Abbreviations: ANMS, 5-azido-1-naphthalene(mono)sulfonate; ANDS, 3-azido-2,7-naphthalenedisulfonate; $NaDodSO_4$, sodium dodecyl sulfate.

of label into the polypeptide subunits of the cytochrome *c* oxidase was accomplished by placing the gel slabs directly on an ultraviolet light source (Ultraviolet Products, Inc., Chromato-vue Transilluminator Model C-63) and observing the fluorescently labeled bands. For a permanent record of the labeling pattern, photography was carried out in the dark with Kodak Plus-X 4 × 5 in. sheet film, ASA 125, through a Kodak 2E filter used to remove the ultraviolet light. Exposure times were 15–30 s, and the film was developed in Kodak DK-50 developer according to the manufacturer's instructions. Radiolabeled samples run on slab gels were sliced into 1.0-m slices and subjected to scintillation counting after solubilization according to procedures (Dockter et al., 1978).

Fluorescence Measurements. All fluorescence measurements were carried out at 25 °C. Steady-state fluorescence spectra were measured on a Perkin-Elmer MPF 44B fluorescence spectrophotometer equipped with a differential corrected spectra microprocessor with rhodamine B as a reference standard. Steady-state anisotropy values were measured by inserting polarizers into the excitation and emission beams and calculating the anisotropy, correcting for the differential transmission characteristics of the spectrophotometer to polarized light according to the procedure of Azumi & McGlynn (1962). Fluorescence nanosecond lifetime measurements were performed on an Ortec Model 9200 nanosecond fluorescence spectrophotometer interfaced to a Hewlett-Packard Model 9845T computer. The lifetime measurements were measured by using a free-running air lamp which excited the sample through a Corning 7-60 excitation filter, and emission was measured through a Schoeffel GM100 grating monochromator set to 500 nm. The computer was used to deconvolute the lamp pulse from the fluorescence measurements by the method of moments (Bay, 1950). Stern–Volmer quenching constants were calculated from linear regression analysis of fluorescence data corrected for dilution (Lehrer, 1971). Fluorescence quantum yields were calculated from the corrected fluorescence emission spetra with 8-anilino-1-naphthalenesulfonate in ethanol (quantum yield = 0.37) as a standard (Wu & Stryer, 1972).

Lactoperoxidase-Catalyzed Iodination. Lactoperoxidase-catalyzed iodination (Phillips & Morrison, 1971) of detergent-solubilized cytochrome *c* oxidase was carried out at 4 °C with 0.1 mM lactoperoxidase and 0.2 mCi of carrier-free ^{125}I per mL in the same buffers used for photolabeling. Iodination was accomplished by 10 additions of 2 mM H_2O_2 (final concentration 5 μM) at 1-min intervals. After iodination, the oxidase was removed from free iodide and Tween 80 detergent by passage through Sephadex G-100 containing 10 mM NaP_i, pH 7.4, buffer containing 2% sodium cholate. Autoradiography was carried out on ^{125}I-labeled cytochrome *c* oxidase after the electrophoresed slab gels had been dried on filter paper by using Kodak X-Omat XR-5 film and developing on a commercial Kodak X-ray film developing machine.

Miscellaneous Procedures. Published procedures were employed for both sodium dodecyl sulfate/polyacrylamide gel electrophoresis systems employed (Douglas et al., 1979; Cabral & Schatz, 1979), protein determinations (Lowry et al., 1951), and heme *a* to protein ratio measurements (Mason & Schatz, 1973). Cytochrome *c* oxidase activity was assayed by the spectrophotometric method (Brautigan et al., 1978). Reduced minus oxidized absorption spectra were obtained on an Aminco DW2 spectrophotometer.

Results

Hydrophobic and Hydrophilic Fluorescent Azides as Probes of Protein Structure. The fluorescent azide 3-azido-2,7-naphthalenedisulfonate is a highly charged and water-soluble molecule. Previous studies (Dockter, 1979) using this probe have shown it to be very useful in the identification of surface polypeptides on intact human erythrocytes since it is too charged to penetrate across the plasma membrane. It is obvious that hydrophobic aryl azides can also be synthesized that, like 3-azido-2,7-naphthalenedisulfonate, have the useful fluorescent properties associated with the naphthalenesulfonic acids. A second fluorescent azide, namely, 5-azido-1-naphthalenesulfonate, was therefore synthesized and predicted to be hydrophobic due to its amphipathic nature. The structures of these probes and the proposed way that they would interact with membranes are shown in Figure 1. The highly charged ANDS would be localized completely in the aqueous phase of the membrane suspension while the apolar end of the 5-azido-1-naphthalenesulfonate would allow it to partition into the hydrophobic region of the membrane.

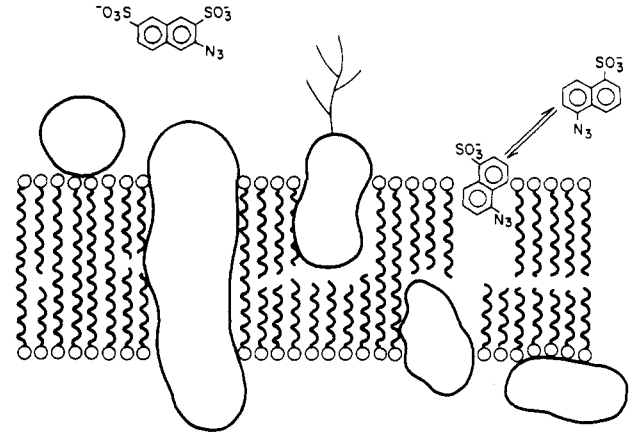

FIGURE 1: Proposed interaction sites of ANDS and ANMS with the hydrophilic and hydrophobic domains of membrane systems.

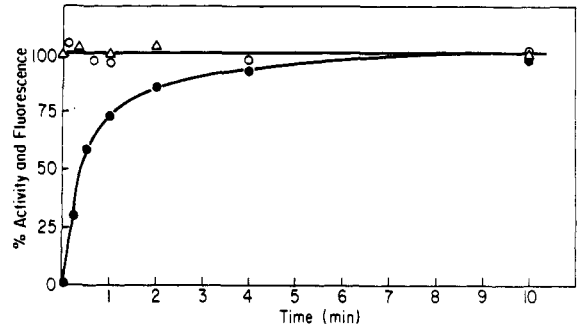

FIGURE 2: Time course of the photolysis of ANDS and its effect on oxidase activity. ANDS was dissolved in the dark in a purified cytochrome *c* oxidase–Tween 80 buffer. At time zero, photolysis was initiated and monitored as an increase in fluorescence (●). At various time intervals, samples were removed into the dark and assayed for catalytic activity (○). Dark controls (△) were also measured which were handled in an identical manner with photolysis sample except they were not exposed to light.

Both ANDS and ANMS are nonfluorescent until photolysis due to the π to π^* transition the azide group can undergo. The products of the photolysis reaction, however, are highly fluorescent, being amino-substituted naphthalenesulfonates. This allows the photolysis reactions to be conveniently monitored by following the development of fluorescence. As seen in Figure 2, under the continuous irradiation techniques employed to photolyze ANDS in the presence of cytochrome *c* oxidase solubilized by Tween 80 detergent, the reaction is essentially complete in 10 min. The effects of this low-flux, long-wavelength, ultraviolet irradiation on cytochrome *c* oxidase activity can be seen to be negligible, either in the presence

Table I: Fluorescence Characteristics of ANDS and ANMS Photolyzed in the Presence and Absence of Detergent

	ANDS		ANMS	
	NaP_i buffer	1% Tween-NaP_i buffer	NaP_i buffer	1% Tween-NaP_i buffer
corrected fluorescence emission maximum (nm)	448	448	578	545
relative quantum yield[a]	1.00	1.00	0.08	1.00
fluorescence lifetime (ns)[b]	19.0	19.0	<3.0	5.6
anisotropy[c]	0.00	0.00	0.01	0.16
Stern-Volmer constant (M^{-1})				
acrylamide	67.6	67.6		
iodide	0.69	0.72	3.20	0.11

[a] Actual quantum yield of ANDS photolyzed in H_2O is 0.29. Actual quantum yield for ANMS was undetermined. [b] Fit to a single-exponential decay, 500-nm emission wavelength. [c] Excitation–emission wavelength pairs; 250 → 468 nm for ANDS and 350 → 550 nm for ANMS.

or in the absence of ANDS. Results with ANMS were essentially identical.

Fluorescence Characteristics of Photolyzed ANDS and ANMS. The fluorescent properties of ANDS and ANMS photolyzed in the presence and absence of detergent can be most useful in determining the localization of the probes with respect to hydrophilic and hydrophobic domains of detergent suspensions. This is, in part, because the fluorescence characteristics of substituted naphthalenesulfonic acids are sensitive to the polarity of their environment and also because the lifetime of these probes is sufficiently long to study physical interactions during their excited state. Table I shows a composite of a number of fluorescence parameters of the photolysis products of ANDS and ANMS in the presence and absence of cytochrome *c* oxidase–detergent solutions. The fluorescent characteristics of the photolysis products of ANDS are unaffected by the presence of Tween 80 in solution with respect to its emission spectrum, quantum yield, and fluorescence lifetime. The physical characteristics of the photolysis products of ANDS are known to be sensitive to polarity of the solvent (Moreland & Dockter, 1980). For example, when the aqueous photolysis products of ANDS examined in Table I are placed in a less polar solvent such as 60% ethanol, the corrected fluorescence emission maximum decreases from 448 to 440 nm, the fluorescence lifetime decreases from 19.0 to 16.7 ns, and the quantum yield is decreased by 10%. Thus, it is concluded that the vast majority of the probe does not interact with the detergent micelle's hydrophobic domains. This is further substantiated by the low anisotropy values reported in Table I which suggest this fluorophore is very free to rotate in the time scale of the excited state in the presence or absence of detergents. Also, the photolyzed ANDS is equally accessible to two aqueous collision quenchers, iodide and acrylamide, as witnessed by the identity of Stern–Volmer constants in the presence and absence of Tween 80 micelles. The linearity of these Stern–Volmer plots suggests a single domain of reporter fluorophores (Figure 3A).

In sharp contrast to ANDS, ANMS appears to interact strongly with the hydrophobic domains of the oxidase-containing detergent micelles. The presence of the detergent causes dramatic increases in the quantum yield and lifetime of the photolysis products of ANMS. There is also a dramatic blue shift of the fluorescence emission spectrum when photolysis is carried out in the presence of detergent. With fluorophores of the general structure 5-amino-1-naphthalenesulfonic acid, it has been well-documented that spectral changes as seen here clearly suggest that in the presence of oxidase-containing Tween 80 micelles, the probe partitions into the more hydrophobic domains of the detergent micelle and away from the aqueous medium (Hudson & Webber, 1973). This is substantiated by the anisotropy

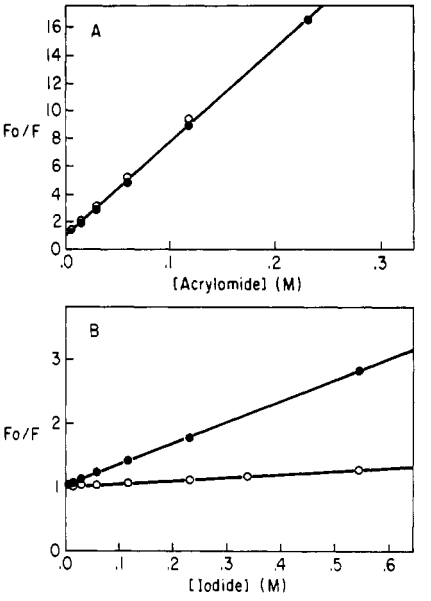

FIGURE 3: Stern–Volmer plots of ANDS and ANMS photolyzed in the presence and absence of Tween 80 micelles. (A) Titration of photolyzed ANDS with increasing acrylamide (quencher) concentration in the presence (○) or absence (●) of 1% Tween 80. (B) Identical with (A) except that photolyzed ANMS was used, and iodide was the quenching reagent.

measurement indicating the flashed ANMS is more immobilized (higher anisotropy regardless of longer lifetime) and is much less accessible to the aqueous collision quencher iodide (Figure 3B). Acrylamide proved to be a very poor quencher for photolyzed ANMS in the presence or absence of detergent. These results substantiate our contention that ANDS photolyzes in the hydrophobic, aqueous domains of detergent-solubilized yeast oxidase while ANMS partitions strongly into the hydrophobic regions of the detergent micelles. Thus, these two labels can be used as topographical probes of these two distinct environments in detergent-solubilized problems such as cytochrome *c* oxidase.

Labeling of Hydrophilic Domains of Yeast Cytochrome c Oxidase. Lactoperoxidase-catalyzed iodination has been the most widely used technique for determining which polypeptides of macromolecular systems are exposed to the aqueous media. Because yeast cytochrome *c* oxidase has previously been surface labeled using the lactoperoxidase-catalyzed iodination technique (Eytan & Schatz, 1975), we repeated this experiment for purposes of comparison with ANDS labeling. Figure 4 compares the stained polypeptide pattern of yeast oxidase to an autoradiogram of the same iodinated gel. Subunits III, VI, and VII contain the bulk of the ^{125}I labeling, with subunits II and IV having lesser amounts. Subunits I and V (which

Table II: Relative Percent Incorporation of Labels into Oxidase Subunits

oxidase subunit	lactoperoxidase-catalyzed ^{125}I[a]	[^{3}H]ANDS[b]	fluorescence		
			ANDS[c]	ANMS[c]	NaDodSO$_4$-ANMS[c,d]
I	<2.0	19.5	21.5	25.2	17.5
II	13.0	13.0	16.0	17.3	9.0
III	20.0	29.0	23.0	29.3	13.4
IV	7.5	14.5	16.0	4.2	12.6
V	<0.5	<1.5	<1.5	<1.5	6.9
VI	28.0	7.5	9.5	<1.5	12.8
VII	29.0	15.0	12.5	21.3	27.9

[a] The relative amount of ^{125}I covalently incorporated into the oxidase polypeptides was obtained by resolving the iodinated oxidase into subunits by a 10%/20% step NaDodSO$_4$/polyacrylamide slab gel electrophoresis, staining the gel with Coomassie blue, destaining, and drying the gel on filter paper followed by autoradiography. The autoradiogram was scanned for density, and the peaks were integrated for total density. [b] Relative amounts of [^{3}H]ANDS incorporated into oxidase polypeptides were determined as in footnote *a* except the gel was cut into 1-mm slices and each slide dissolved and scintillation counted. Counts under peaks were then integrated by simple counting of peak slices. Total incorporation 0.30 mol of [^{3}H]ANDS/mol of oxidase. [c] Relative intensities of fluorescence associated with each oxidase subunit were obtained by scanning densitometry of photographic negatives of fluorescence on gels as described under Materials and Methods. [d] In the NaDodSO$_4$-ANMS sample, the oxidase complex was first disrupted by placing the enzyme in a 1% NaDodSO$_4$ solution before the addition of ANMS and subsequent photolysis.

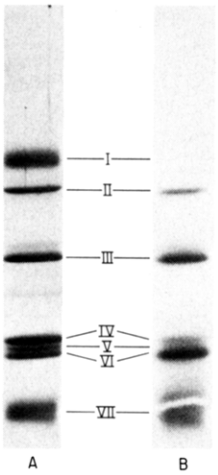

FIGURE 4: Lactoperoxidase-catalyzed iodination of purified, detergent-solubilized yeast cytochrome *c* oxidase. Iodination was carried out as described under Materials and Methods. After separation of polypeptides by 10–17% exponential gradient NaDodSO$_4$/polyacrylamide gel electrophoresis, their location was determined by staining with Coomassie blue (A). After photography, ^{125}I incorporation was determined by drying the gels onto filter paper and subjecting them to autoradiography (B). The Roman numericals correspond to subunit nomenclature of Cabral & Schatz (1979).

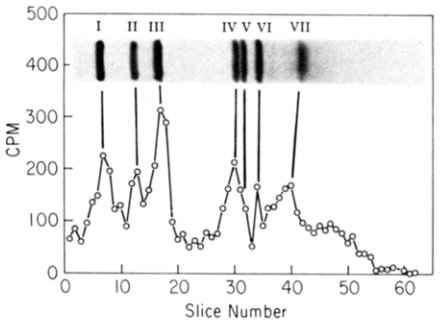

FIGURE 5: Incorporation of [^{3}H]ANDS into yeast cytochrome *c* oxidase subunits by photolysis. Conditions were as described under Materials and Methods except [^{3}H]ANDS was used. After detection of bands on 10/20% step NaDodSO$_4$/polyacrylamide gel electrophoresis by Coomassie blue staining, the gel was cut and counted for radioactivity. Inset shows original gel with the top on the left side.

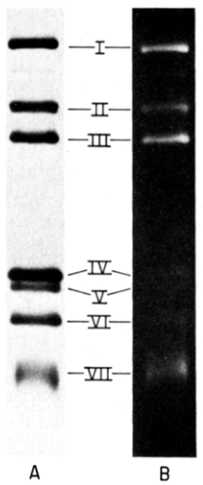

FIGURE 6: Fluorescent detection of ANMS labeling of detergent-solubilized oxidase. Purified, detergent-solubilized cytochrome *c* oxidase was photolyzed in the presence of ANMS. After separation of the polypeptides on a 10/20% step NaDodSO$_4$/polyacrylamide gel electrophoresis, the polypeptides were detected by Coomassie blue staining (A). Incorporation of photolabels into protein was detected by photographing the fluorescence of ANMS-modified polypeptides under ultraviolet irradiation before Coomassie blue staining (B).

can be resolved from subunits IV and VI better on a step gel) contained minimal amounts of radioactivity. Quantitation of these results is shown in Table II.

When ANDS or [^{3}H]ANDS is used to label hydrophilic domains of the purified oxidase, the localization of the label can be conveniently detected on labeled polypeptides by either acquired fluorescence after separation on sodium dodecyl sulfate/polyacrylamide gel electrophoresis or incorporated radioactivity, respectively. Table II compares these two quantitative approaches. It can be seen that in addition to subunits II, III, IV, VI, and VII, subunit I is clearly fluorescently tagged. Subunit V appears labeled but to a much smaller level than the other subunits. For purposes of absolute quantitation, the yeast oxidase was labeled with tritiated ANDS, the subunits were separated on a 10%/20% NaDodSO$_4$/polyacrylamide gel electrophoresis step gel, photographed after polypeptide staining, and cut and counted for associated radioactivity. Figure 5 shows the resulting pattern which confirms the results of the fluorescence photography. All subunits of oxidase contain [^{3}H]ANDS with the possible exception of subunit V which contained only a minimal amount of label. Table II indicates the relative percentages of label incorporated into oxidase subunits by the lactoperoxidase-catalyzed iodination technique compared to the fluorescence incorporated by ANDS photolysis and radiolabel incorporated by [^{3}H]ANDS.

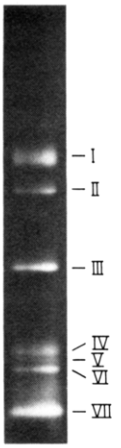

FIGURE 7: Labeling of cytochrome *c* oxidase subunits by the photolysis of ANMS in the presence of $NaDodSO_4$. Labeling was identical with that of Figure 6B except 1% $NaDodSO_4$ replaced the 1% Tween 80 in the buffer.

Labeling of Hydrophobic Domains of Yeast Cytochrome c Oxidase. The incorporation of photolyzed ANMS into the more hydrophobic regions of the oxidase–detergent micelle mixtures results in the incorporation of fluorescent label into the mitochondrially synthesized subunits I–III and the cytoplasmically synthesized subunit VII (Figure 6). Somewhat variable, small levels of label appear in subunits IV, V, and VI. This labeling pattern is quite distinct from the labeling pattern seen with ANDS. The percentages incorporated into each subunit have been quantitated to Table II by integrating areas under peaks of scanning densitometry tracings of the photographic negatives of bound fluorescence on the sodium dodecyl sulfate/polyacrylamide gels. It is clear that well over 90% of the incorporated label can be localized on subunits I, II, III, and VII of yeast cytochrome *c* oxidase. If the photolabeling of yeast oxidase is repeated with ANMS in the presence of sodium dodecyl sulfate used to disrupt subunit–subunit interactions, the labeling is no longer selective as subunits IV, V, and VI also become fluorescently tagged (Table II and Figure 7). This is as expected since each subunit, upon dissociation, should become surrounded by a hydrophobic layer of alkyl chains of $NaDodSO_4$.

Discussion

The two fluorescent azides used in this study show great potential for the topographical labeling of hydrophilic and hydrophobic domains of membrane complexes such as purified yeast cytochrome *c* oxidase. The advantages of using these labels, as opposed to radiolabeling techniques, are low cost, speed of reaction, relative insensitivity to reaction media conditions, and elimination of radioisotopes and related radioisotopic techniques. The unique feature of these new topographical labels is their fluorescence characteristics. ANDS and ANMS have long-wavelength absorption bands such that associated azides can be photolyzed with long ultraviolet wavelengths which are relatively harmless to protein integrity and funtion. Products of photolysis emit in the visible spectrum with relatively high quantum yields. This allows for easy detection of polypeptide bands with covalently bound label via their acquired fluorescence. In addition, the sensitivity of the fluorescence of the naphthalenesulfonates to the polarity of their environment, coupled with long fluorescence lifetimes and high quantum yields, allows the use of fluorescence techniques to sample the environment of the photolyzed probes. By use of these techniques, it has been shown that 3-azido-2,7-naphthalenedisulfonate is localized and photolyzes in the aqueous hydrophilic domains of a solution of yeast cytochrome *c* oxidase solubilized in Tween 80 detergent. It comes in close enough proximity to six of the seven subunits of yeast cytochrome *c* oxidase to covalently modify them. Subunit V appears little, if at all, accessible to ANDS. 5-Azido-1-naphthalenesulfonate, on the other hand, can be shown to be sequestered and photolyzed in the hydrophobic milieu of the protein–detergent micelle complex. Because of the amphipathic nature of ANMS, this positioning is most probably near the water–detergent interface region with the charged sulfonate group protruding out and the naphthyl azide sequestered inside the micelle in a fashion similar to that believed to be true for 8-anilino-1-naphthalenesulfonate (Radda & Vanderkooi, 1972). Stern–Volmer quenching studies indicate that the label is largely protected from external aqueous quenchers. As a probe of hydrophobic regions of detergent-solubilized oxidase, ANMS is capable of covalently modifying subunits I, II, III, and VII, with subunits IV–VI incorporating relatively minimal amounts of label.

How do the results presented here with ANDS and ANMS compare to previous studies on the topography of cytochrome *c* oxidase? Topographical labeling of the purified yeast oxidase under similar conditions has been carried out by using the surface-labeling techniques of lactoperoxidase-catalyzed iodination and labeling with *p*-benzenediazoniumsulfonate (Eytan & Schatz, 1975). The present results are in good agreement with these earlier results with one notable exception. Purified oxidase was labeled in the subunit I region of the $NaDodSO_4$/polyacrylamide gel by lactoperoxidase-catalyzed iodination. Our iodination procedure did not incorporate significant amounts of label into subunit I. However, photolyzed ANDS did incorporate label into subunit I. It is known that the earlier preparations of yeast cytochrome *c* oxidase often carried high molecular weight contaminants which may have comigrated with subunit I on gels and contributed to the labeling of the subunit I region. In this work, the differential labeling of subunit I between iodination and ANDS incorporation could be due to the greater accessibility of the ANDS to some hydrophilic surface on subunit I or the lack of appropriate nucleophiles (generally tyrosines) on the exposed surface of subunit I for iodination to take place. In addition to the above difference between earlier surface-labeling studies and this work, it is now possible to clearly resolve subunits IV, V, and VI by $NaDodSO_4$/polyacrylamide gel electrophoresis. The interesting result emerges that yeast subunit V, a cytoplasmically synthesized subunit, is not readily accessible to surface labeling by iodination or ANDS photolysis, nor is it accessible to hydrophobic labels. This is of interest because it has been suggested that the cytoplasmic subunits constituted the bulk of the hydrophilic domains of the complex while the mitochondrially synthesized subunits comprised the more hydrophobic core of the enzyme and interacted predominantly with the membrane lipid (or detergent in this case). Subunit V appears not to be inaccessible to any of these probes, suggesting it is buried deep within the core of the complex. Further information on this point may have to await amino acid and sequence analysis on this subunit.

The labeling of mitochondrially synthesized subunits I, II, and III and cytoplasmically synthesized subunit VII by the hydrophobic ANMS is in excellent agreement with the results of Cerletti & Schatz (1979), who used the radiolabeled lipophilic aryl azides 5-iodonaphthyl 1-azide and 5-[4-azido-2-nitrophenyl]thiophenol to study hydrophobic domains of yeast oxidase as well as more recent studies using aryl azide analogues of phospholipids by Gutweniger et al. (1981). Both of

these previous studies noted a small but significant amount of labeling in subunit V not seen with AMNS. The inability of AMNS to label subunit V most likely reflects the depth at which these aryl azides reach into the hydrophobic domain.

Comparisons can also be made between the results presented here on the yeast oxidase to topographical studies published on bovine cytochrome *c* oxidase since these two complexes are compositionally very similar (Azzi, 1980). In particular, direct comparisons can be made between subunits I, II, III, and VI of the yeast enzyme and the corresponding subunits I, II, III, and V of the beef enzyme, where sequence homology can be directly demonstrated (Bonitz et al., 1980; Anderson et al., 1982; Coruzzi & Tzagoloff, 1979; Fox, 1979; Thalenfeld & Tzagoloff, 1980; Gregor & Tsugita, 1982; Tanaka et al., 1979). Labeling of the hydrophilic domains of bovine oxidase using the water-insoluble protein-modifying reagent *p*-benzenediazonium[^{35}S]sulfonate ([^{35}S]DABS) (Eytan et al., 1975; Eytan & Broza, 1978; Ludwig et al., 1979; Prochaska et al., 1980) or antibodies directed against specific subunits (Chan & Tracy, 1978) indicates that subunits II, III, IV, V, and VII are all exposed to the hydrophilic media. Subunit I and VI of the beef enzyme were not labeled to any significant extent. These results are in agreement with the lactoperoxidase labeling results presented here if one assumes that subunit V of the yeast enzyme corresponds to subunit VI of the beef enzyme. The use of aryl azides to study the topography of both the hydrophilic and hydrophobic domains of the beef oxidase has been reported in an interesting study by Prochaska et al. (1980). Hydrophobic domains of the beef oxidase were photolyzed in the presence of radiolabeled aryl azidophospholipids. The incorporated label was associated predominantly with subunits I, II, III, and VII, in agreement with similar studies on the yeast oxidase (Gutweniger et al., 1981) and our labeling pattern with AMNS. Labeling of the hydrophilic domains of the beef oxidase by flash photolysis was accomplished by using 2-[(4-azido-2-nitrophenyl)amino]-ethane[^{35}S]sulfonate (NAP-taurine) as a surface label. Prochaska et al. (1980) found that all beef oxidase subunits incorporated NAP-taurine. This was in contrast to their finding that [^{35}S]DABS did not evaporate significant label into subunits I and VI of the beef enzyme [see, however, an earlier report from this laboratory in which significant amounts of label were incorporated into subunit I by [^{35}S]DABS under similar labeling conditions (Ludwig et al., 1979)]. These authors have also suggested that the additional labeling by the NAP-taurine on subunits not labeled by DABS may be due to the lack of specific nucleophiles on the exposed surfaces of these subunits that are needed for DABS reaction to take place. It should be noted here that NAP-taurine has recently been shown to have the potential to interact with lipid bilayers or detergents due to its amphipathic nature (Richards & Brunner, 1980). Thus, this reagent is not rigorously suitable, a priori, as a photolabile surface reagent, and appropriate care must be taken to ensure that it is reacting predominantly in hydrophilic domains under a given set of conditions [see, for example, Prochaska et al. (1980)].

When the data presented here are combined with the earlier labeling studies of purified detergent-solubilized yeast oxidase, the following general conclusions can be drawn. First, all subunits are accessible to hydrophilic reagents except for subunit V which apparently is a subunit sequestered deep within the complex's core. Second, subunits I, II, III, and VII are localized in hydrophobic domains of the complex. The fluorescent azides utilized in this study should remain useful in further studies to determine the proximity of their binding domains to endogenous heme *a* and the cytochrome *c* binding site as well as in situ topographical studies of other oxidase membrane proteins.

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Registry No. ANMS, 73105-93-8; ANDS, 70476-18-5; cytochrome *c* oxidase, 9001-16-5; 3-amino-2,7-naphthalenedisulfonic acid, 92-28-4.

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Primary Structure of Papain-Solubilized Human Histocompatibility Antigen HLA-B40 (-Bw60). An Outline of Alloantigenic Determinants†

José A. López de Castro, Rafael Bragado,* Douglas M. Strong, and Jack L. Strominger

ABSTRACT: The primary structure of papain-solubilized human histocompatibility antigen HLA-B40 (-Bw60) has been determined. Its comparison with that of the cross-reactive HLA-B7 antigen allows for the first time a direct sequence comparison between two HLA-B locus products and an outline of the location of their alloantigenic sites. Overall sequence homology between HLA-B40 and HLA-B7 is 93%. Of 19 detected differences, 18 are located in the two amino-terminal domains (residues 1–182). Half of them are clustered in two short segments spanning residues 63–74 and 147–156, respectively, which suggests that they may encompass major sites of their alloantigenic determinants. The first of these segments is highly polymorphic in HLA and H-2 molecules. It is proposed that it may belong to a hypervariable region of class I histocompatibility antigens. The remaining substitutions are scattered through the N-terminal portions of the two external domains. Thus, in addition to the above-mentioned segments, other positions may contribute significantly to the antigenic polymorphism of these molecules.

Human class I histocompatibility antigens are a family of integral membrane glycoproteins encoded by the HLA-A, -B, and -C loci of the major histocompatibility complex (MHC).[1] They are ubiquitously distributed on the surface of the great majority of cells and appear to play a central role in mediating the recognition and killing of virus-infected or otherwise modified cells by specific cytotoxic T lymphocytes (McMichael et al., 1977; Dickmeiss et al., 1977). Serological analyses of class I HLA antigens have revealed an extraordinary degree of genetic polymorphism. This polymorphism is a consequence of the existence of three loci which encode for class I molecules, each locus containing one of a great number of alleles (Albert et al., 1980) which are codominantly expressed. The functional meaning of this diversity is obscure, although it has been proposed that it may be important in maximizing the repertoire of associative recognition of foreign antigens expressed on the surface of target cells (Kimball & Coligan, 1983).

Biochemical characterization of HLA-A and -B antigens has established that the molecule consists of a polymorphic, MHC-encoded heavy chain of 44 000 daltons noncovalently bound to β_2-microglobulin (Grey et al., 1973), an invariant 12 000-dalton polypeptide encoded outside the MHC (Goodfellow et al., 1975). The heavy chain consists of a large amino-terminal extracellular portion, a transmembrane hydrophobic segment, and a carboxy-terminal hydrophilic portion which is in contact with the cytoplasm (Springer & Strominger, 1976). Primary structural analysis suggests that the extracellular portion may be organized in 3 domains of approximately 90 residues each (Orr et al., 1979), which have been designated as α1 (residues 1–90), α2 (residues 91–182), and α3 (residues 183–275). Papain treatment of cell membranes solubilizes the extracellular portion of the molecule (HLA_{pap}), which includes the antigenic determinants (Sanderson & Batchelor, 1968; Springer et al., 1974). Papain-solubilized HLA antigens are suitable for large-scale purification (Parham et al., 1977; Trägård et al., 1979), and for this reason, they have been used for most studies dealing with detailed structural analyses of these molecules (Strominger et al., 1980). A major goal of these studies is to provide a

† From the Department of Immunology, Fundación Jiménez Díaz, Avenida Reyes Católicos, 2 Madrid-3, Spain (J.A.L.d.C. and R.B.), the Naval Medical Research Center, Bethesda, Maryland 20814 (D.M.S.), and the Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Massachusetts 02138 (J.L.S.). *Received February 14, 1983.* Supported by grants from the Spanish Ministerio de Educación y Ciencia (3448-79), from the Fondo de Investigaciones Sanitarias de la Seguridad Social (10/81 and 12/81), and from the National Institutes of Health (A1-10736). This work was also supported in part by the Naval Medical Research and Development Command (Work Unit M0095-PN.001-0045) and by Contract Y001CP00502 from the National Cancer Institute. R.B. was a fellow of the Spanish Fundación Jiménez Díaz.

[1] Abbreviations: MHC, major histocompatibility complex; HLA_{pap}, papain-solubilized HLA antigen; $NaDodSO_4$, sodium dodecyl sulfate; CNBr, cyanogen bromide; HPLC, high-pressure liquid chromatography; Pth, phenylthiohydantoin; CTL, cytotoxic T lymphocyte.

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