Subunit Dissociation and Protein Unfolding in the Bovine Heart Cytochrome Oxidase Complex Induced by Guanidine Hydrochloride

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Received June 19, 1987; Revised Manuscript Received January 8, 1988

ABSTRACT: The response of cytochrome oxidase to the denaturant guanidine hydrochloride (Gdn·HCl) occurs in two stages. The first stage is a sharp transition centered at 1 M Gdn·HCl, whereas the second stage occurs from 3 to 7 M Gdn·HCl. In the first phase, changes occur in several spectroscopic properties: (1) the tryptophan fluorescence increases from 37% of that of *N*-acetyltryptophanamide to 85%; (2) the emission maximum shifts from 328 to 333 nm; (3) the circular dichroism (CD) signal at 222 nm diminishes by 30%; and (4) the Soret CD signal at 426 nm is completely abolished. These spectroscopic changes are accompanied by complete loss of the oxidase's steady-state electron-transfer activity. Of the 13 available sulfhydryl residues, 2 are reactive in the isolated enzyme, but this number increases to almost 10 in the first stage of denaturation. Subunits III, VIb, VIc, and VII dissociate from the protein complex at 0.5 M Gdn·HCl, but only subunit VII can be recovered after gel filtration chromatography [nomenclature according to Buse et al. (1985)]. In 2.5 M Gdn·HCl, the heme groups are found with a complex consisting predominantly of subunits I, II, and IV. In the second phase of denaturation, there is further disruption in the structure of the oxidase as indicated by continued decline in the ultraviolet CD signal and shift to longer wavelength of the tryptophan emission spectrum. However, the fluorescence quantum yield and number of reactive sulfhydryl groups decrease as the denaturant level is raised. Gel filtration chromatography reveals that protein and heme form a high molecular weight aggregate at 5 M Gdn·HCl. These data indicate that cytochrome oxidase contains two regions of differing sensitivity to Gdn·HCl. The region of highest sensitivity, most likely the C domain, appears to be the subunits associated with the heme prosthetic groups. The regions most resistant to denaturation are probably the membrane-embedded M_1 and M_2 domains.

Cytochrome oxidase (EC 1.9.3.1) is a member of the mitochondrial respiratory chain and functions to catalyze the transfer of reducing equivalents from cytochrome *c* to O_2 and to conserve some of the free energy of this reaction as an electrochemical gradient across the inner mitochondrial membrane. The complex is composed of 12 or 13 nonidentical protein subunits containing 2 heme A moieties and 2 copper atoms as prosthetic groups (Wikström et al., 1985). The complex is arranged so as to span the inner mitochondrial membrane. Electron diffraction studies of two-dimensional crystals have led to the advancement of a low-resolution structural model for the enzyme complex (Fuller et al., 1979; Deatherage et al., 1982a,b; Frey et al., 1985). The protein subunits are found to be arranged in a Y-shaped structure with the arms protruding through the membrane into the matrix space with the base exposed on the cytoplasmic surface. Three domains are recognized in this structure: two membrane domains, M_1 and M_2, and a cytoplasmic domain, C.

Many denaturation studies of proteins, using such structural perturbants as guanidine hydrochloride and urea, have been performed for both water-soluble [e.g., see Tanford (1968), Visser et al. (1975), Kelly and Stellwagon (1984), and Horami and Oshimi (1979)] and integral membrane proteins [e.g., see Tajima et al. (1976), Rizzolo and Tanford (1978), and Nozaki et al. (1978)]. In general, these two classes of proteins differ greatly in their sensitivity to denaturants. Denaturation of water-soluble proteins proceeds as a cooperative process over a very narrow range of denaturant concentration, whereas integral membrane proteins are typically very resistant to denaturation and in some cases are not fully devoid of structure under the harshest of conditions (Nozaki et al., 1978). Since cytochrome oxidase is proposed to consist of cytoplasmic and integral membrane domains, we reasoned that this tripartite structure might be revealed in a denaturation experiment.

This paper describes the response of cytochrome oxidase to the denaturant guanidine hydrochloride (Gdn·HCl).[1] We observe two phases of denaturation: one at low Gdn·HCl concentration over a narrow range of denaturant concentration and a second phase occurring at high Gdn·HCl concentration that is still not complete in 7 M Gdn·HCl. We suggest that this behavior reflects the multidomain structure of the enzyme; the low Gdn·HCl transition corresponds to the C domain, whereas the transition occurring at high Gdn·HCl concentration arises from the M domains. The low-concentration Gdn·HCl transition is associated with the complete loss of the Soret CD signal and indicates a C domain location for the heme groups in the enzyme.

Materials and Methods

Guanidine hydrochloride was extreme purity grade obtained from Heico Division of Whittaker Corp. (Delaware, PA). Lauryl maltoside was purchased from Calbiochem. Acrylamide was the twice-recrystallized variety obtained from Serva. Bis(acrylamide) was from Kodak. Sodium dodecyl sulfate was from BDH Chemicals. Tris was purchased from Bethesda Research Laboratories.

Cytochrome oxidase was made according to the method of Kuboyama et al. (1972) except that the enzyme was ammo-

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[1] Abbreviations: Gdn·HCl, guanidine hydrochloride; CD, circular dichroism; UV, ultraviolet; SDS, sodium dodecyl sulfate; NATA, *N*-acetyltryptophanamide; PDS, 4,4′-dipyridyl disulfide; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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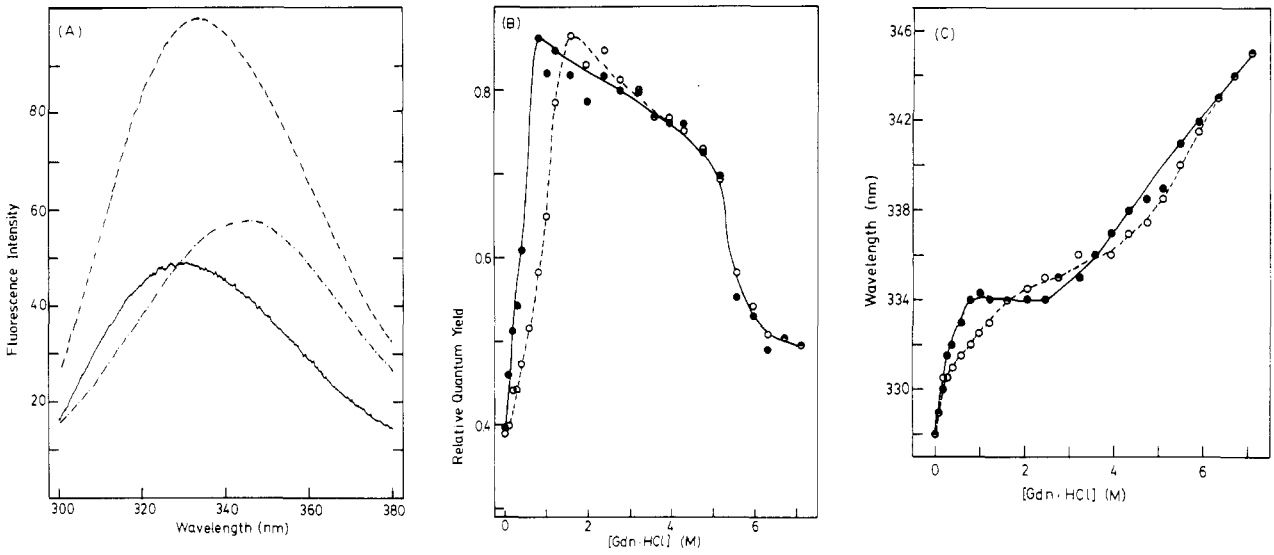

FIGURE 1: Effect of Gdn·HCl concentration on the intrinsic tryptophan fluorescence of cytochrome oxidase. Samples of cytochrome oxidase at a concentration of 1 mg of protein/mL were prepared in 0.02 M Tris, 0.1 M NaCl, 1 mg/mL lauryl maltoside, and 1 mM EDTA, pH 7.8, and the specified concentration of Gdn·HCl. Fluorescence spectra were measured after incubation for 24 h at 25 °C at a protein concentration of 0.043 mg/mL in the same buffer. (A) Gdn·HCl concentration was 0 (—), 1 (- - -), and 7.15 M (–·–). (B) Fluorescence intensity of the intrinsic tryptophan fluorescence of cytochrome oxidase as a function of Gdn·HCl concentration. Quantum yield was determined at 1 (○) and 24 h (●) relative to NATA by integration of the emission intensity between 300 and 420 nm. (C) Emission maximum of the intrinsic tryptophan fluorescence of cytochrome oxidase plotted against Gdn·HCl concentration. The samples were measured after 1- (○) and 24-h (●) incubation at 25 °C.

nium sulfate fractionated in buffer containing sodium cholate and the final pellet was dissolved in 25 mM sodium phosphate pH 7.4, with 1 mg/mL lauryl maltoside. The protein was stored at −70 °C at a concentration of 40 mg/mL.

Stock solutions of 8–10 M Gdn·HCl were prepared in H_2O. The pH was adjusted to between 7 and 8 and the concentration determined by density. Gdn·HCl was diluted into buffer to obtain the desired denaturant concentration. Tris buffer (20 mM, pH 7.8) with 1 mg/mL lauryl maltoside, 1 mM EDTA, and 0.1 M NaCl was used in all experiments.

Fluorescence measurements were made on a Perkin-Elmer MPF 44A fluorometer operated in the ratio mode. The excitation wavelength was 280 nm, and the band-pass of both emission and excitation monochromators was 6 nm. The temperature for the fluorescence determinations was 25 °C. Quantum yield values were estimated by reference to the fluorescence of the tryptophan analogue *N*-acetyltryptophanamide in the same solution as the enzyme. The emission intensities were obtained by integration of the emission profiles and were corrected for the inner filter effect at the wavelength of excitation (Lakowicz, 1983) and normalized to the same absorbance at 280 nm.

Circular dichroism spectra were recorded on a Jasco J500C spectropolarimeter. Spectra were recorded at 25 °C and a bandwidth of 2 nm. In the ultraviolet region, the path length was 0.05 cm, whereas in the Soret region it was 1 cm.

The steady-state electron-transfer activity of cytochrome oxidase was measured by monitoring the oxidation of reduced cytochrome *c* spectrophotometrically at 550 nm. This assay was done in 25 mM sodium phosphate buffer at pH 7.0 with 1 mM EDTA and 1 mg/mL lauryl maltoside. The assay was initiated by addition of the oxidase, and the end point was determined by addition of potassium ferricyanide. Reduced cytochrome *c* was prepared by incubation of a concentrated solution of the protein (i.e., ≃5 mM) with 50 mM sodium ascorbate and then separating this mixture by chromatography on a column of G-25 Sephadex.

Absorbance spectra were obtained on a Cary 219 UV–visible spectrophotometer. The spectra were measured at 25 °C with a bandwidth of 2 nm in a cuvette with a path length of 1 cm. Absorbance measurements were also made on a Zeiss PM6 spectrophotometer.

Gel chromatography was performed using a matrix of Sephacryl S-200 that was equilibrated in buffer at the desired concentration of Gdn·HCl. The column (0.9 × 52 cm) was eluted at a flow rate of 10 mL/h. The eluant was passed sequentially through a Gilson Holochrome monitor set at 420 nm and an LKB monitor measuring absorbance at 280 nm. The outputs from these monitors were displayed on two stripchart recorders. The flow rate was controlled by a Gilson minipulse peristaltic pump, and fractions were collected by using a Gilson microfractionator.

Subunit content of fractions obtained from the S-200 column was assessed by electrophoresis in 15% acrylamide gels with 0.1% SDS and 2 M urea as described by Robinson et al. (1980). The samples were prepared for electrophoresis by dialysis to remove Gdn·HCl and then dialyzed in Tris–phosphate buffer at pH 6.8 after addition of 2% SDS. The oxidase subunits are designated according to the nomenclature of Buse et al. (1985).

RESULTS

The response of the intrinsic tryptophan fluorescence of cytochrome oxidase to increasing Gdn·HCl concentration is shown in Figure 1. Three representative fluorescence emission spectra are shown in Figure 1A. In buffer without Gdn·HCl, the enzyme has an emission peak at 328 nm. The quantum yield is 37% relative to *N*-acetyltryptophanamide in the same buffer. This spectrum is compared to those obtained in 1 M Gdn·HCl and 7.15 M Gdn·HCl. In 1 M Gdn·HCl, the emission spectrum of the oxidase has shifted to 334 nm and has an intensity 85% that of the tryptophan analogue under the same conditions. At 7.15 M Gdn·HCl, the emission maximum has shifted to 346 nm, but the intensity has fallen to about 50% that of NATA. Figure 1B,C illustrates the response of the fluorescence intensity and emission maximum as a function of Gdn·HCl concentration at 1-h exposure and after 24-h exposure of the protein to Gdn·HCl. When exam-

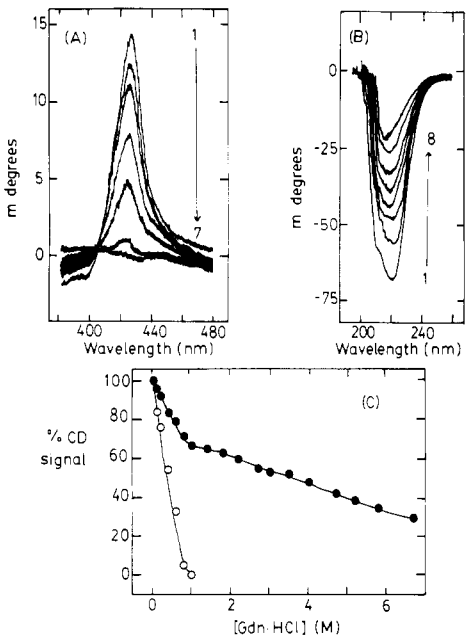

FIGURE 2: Effect of Gdn·HCl concentration on the UV and Soret circular dichroism of cytochrome oxidase. Cytochrome oxidase was at a concentration of 1 mg of protein/mL. Circular dichroism measurements were made at a bandwidth of 2 nm, a time constant of 0.5 s, and a scan rate of 20 nm/min. (A) Circular dichroism spectra of cytochrome oxidase in the Soret region at different concentrations of Gdn·HCl. The spectra are arranged sequentially 1–7 and correspond to the following Gdn·HCl concentrations: (1) 0, (2) 0.1, (3) 0.2, (4) 0.4, (5) 0.6, (6) 0.8, and (7) 1.0 M. (B) Circular dichroism spectra in the UV region of cytochrome oxidase at different concentrations of Gdn·HCl. The spectra are arranged sequentially 1–8 and correspond to the following concentrations of Gdn·HCl: (1) 0, (2) 0.4, (3) 0.8, (4) 1.4, (5) 2.2, (6) 3.5, (7) 5.2, and (8) 6.7 M. (C) Circular dichroism signal strength as a function of Gdn·HCl concentration. The signal intensities were normalized to 100% at 0 Gdn·HCl at 222 nm (●) and at 426 nm (○).

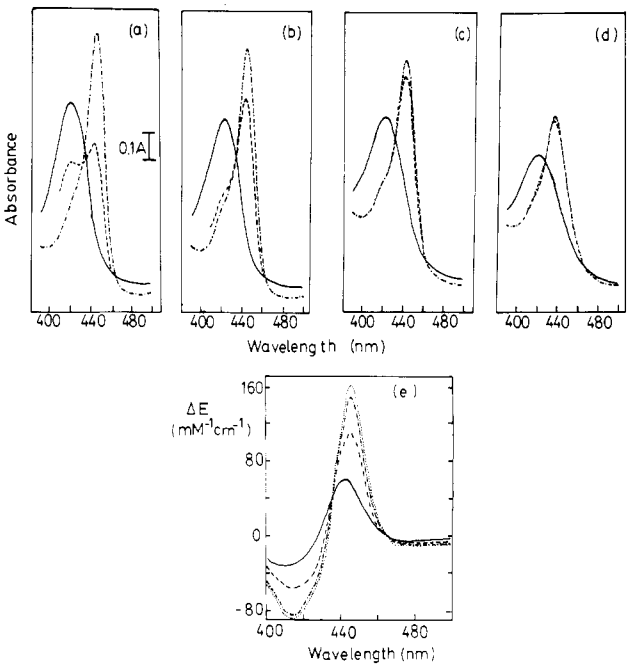

FIGURE 3: Effect of Gdn·HCl concentration on the absorbance spectra of cytochrome oxidase in the Soret region. Cytochrome oxidase (1.18 mg/mL) was incubated at different levels of Gdn·HCl. The concentrations of Gdn·HCl used were (a) 0, (b) 0.1, (c) 0.4, and (d) 1.0 M. Spectra were recorded of the oxidized enzyme (—), partially reduced enzyme obtained immediately after dithionite addition (i.e., <1 min) (- - -), and fully reduced enzyme 20 min after dithionite addition (–·–). The absorbance scale used throughout is indicated on panel a. Panel e shows the difference spectra calculated for the fully reduced – oxidized species at the following Gdn·HCl concentrations: 0 (···), 0.1 (–·–), 0.4 (- - -), and 1.0 M (—).

ined after 1-h exposure to Gdn·HCl, the fluorescence intensity of the oxidase increases from about 37% to 85% of NATA as the Gdn·HCl concentration is increased from 0 to 2 M, indicating the movement of tryptophan away from the quenching heme centers. Above 2 M Gdn·HCl, the intensity decreases until it is about 50% of NATA at 7 M Gdn·HCl. When examined after 24-h incubation, a similar pattern is observed except the initial increase in fluorescence intensity is shifted to lower Gdn·HCl concentration and the maximum intensity occurs at 1 M Gdn·HCl. No further change is observed after longer periods of incubation. In Figure 1C, the fluorescence emission maximum is plotted as a function of Gdn·HCl concentration after 1- and 24-h incubation periods. The wavelength maximum of the fluorescence emission increases from 328 to 345 nm between 0 and 7 M Gdn·HCl which indicates the transfer of fluorescently visible tryptophans from a nonpolar to a polar environment. After 1-h incubation, the response is biphasic with the first phase complete at 2 M Gdn·HCl and the second phase extending from 2 to 7 M Gdn·HCl. This biphasic behavior becomes more pronounced after incubation for 24 h in Gdn·HCl. The first phase response is shifted to a lower denaturant concentration and is complete at 1 M Gdn·HCl. The first phase is well separated from the second which begins at approximately 2 M and is not complete at 7 M Gdn·HCl.

In Figure 2, the response of cytochrome oxidase to Gdn·HCl concentration is shown as monitored by circular dichroism in the ultraviolet and Soret spectral regions. In the Soret region, cytochrome oxidase has a CD signal centered at 426 nm (Figure 2A). This band decreases uniformly in intensity as the Gdn·HCl concentration is increased from 0 to 1 M, indicating the disruption of the asymmetric environment surrounding the hemes. In the ultraviolet region, the oxidase exhibits a signal of negative CD intensity at 222 nm (Figure 2B). The strength of this signal, which monitors the secondary structure, slowly diminishes in intensity and shifts to 217 nm as the concentration of Gdn·HCl is increased from 0 to 6.7 M. Figure 2C shows a plot of the CD intensity in both regions (426 and 222 nm) as a function of increasing Gdn·HCl concentration. At 222 nm, the biphasic response of the oxidase to the denaturant is evident. In contrast, only a single phase of denaturation can be followed in the Soret region. It occurs between 0 and 1 M Gdn·HCl and results in the complete loss of this signal. These results were obtained with samples incubated for 24 h. Samples incubated for 1 h revealed a similar biphasic pattern with the first phase shifted to higher Gdn·HCl concentration as was seen with the fluorescence results.

Figure 3 shows absorbance spectra of cytochrome oxidase obtained in the Soret region over the concentration range in which we observe loss of the Soret CD signal, 0–1 M Gdn·HCl. In panel a, the spectra of oxidized, partially reduced, and fully reduced cytochrome oxidase are shown between 400 and 500 nm. Our preparation of the oxidized enzyme in the absence of Gdn·HCl has its spectral maximum at 418 nm. Addition of dithionite in the absence of Gdn·HCl initially reveals a spectrum with two peaks representing enzyme with nearly fully reduced cytochrome *a* and nearly completely oxidized cytochrome a_3. The partially reduced enzyme slowly converts to the fully reduced species as the cytochrome a_3 is slowly reduced with dithionite. We have repeated the same protocol for the enzyme incubated for 24 h at 0.1, 0.4, and 1.0 M Gdn·HCl (see panels b, c, and d, respectively). The spectrum of the oxidized enzyme undergoes a shift to longer wavelength as the

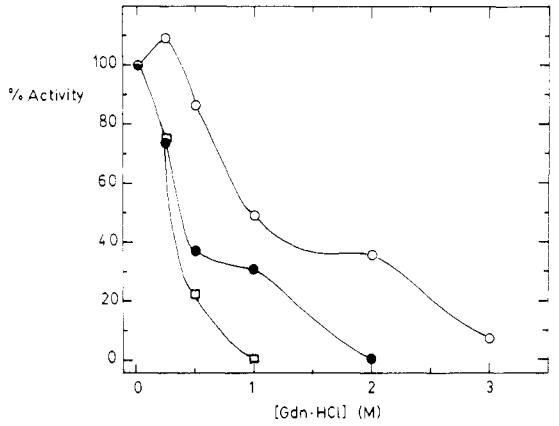

FIGURE 4: Effect of Gdn·HCl concentration on the steady-state electron-transfer activity of cytochrome oxidase. Cytochrome oxidase was incubated at a concentration of 2 mg/mL in 20 mM Tris buffer, pH 7.8, with 1 mM EDTA, 1 mg/mL lauryl maltoside, and varying concentrations of Gdn·HCl. The enzyme was diluted 1:10 into 20 mM Tris, pH 7.8, with 1 mM EDTA, 5 mg/mL lauryl maltoside, and 100 mM NaCl and then a further 150 times into the assay buffer at different times following initial exposure to Gdn·HCl: (○) immediately; (●) 1 h; and (□) 24 h. The assay was done in 800 μL of 25 mM sodium phosphate, pH 7.0, with 1 mM EDTA and 1 mg/mL lauryl maltoside at 25 °C. Reduced cytochrome *c* was added to a concentration of 28 μM prior to the addition of the oxidase at a concentration of 6 nM. The maximal electron-transfer activity of our preparation was 200 electrons s^{-1} oxidase $^{-1}$.

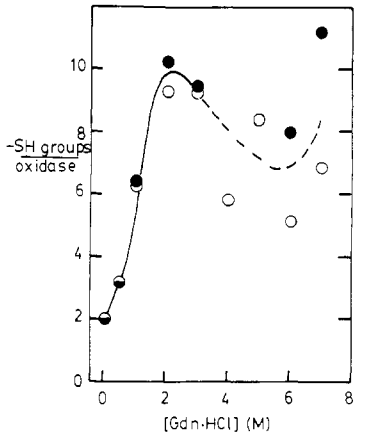

FIGURE 5: Titration of cytochrome oxidase with the sulfhydryl reagent 4,4′-dipyridyl disulfide. Cytochrome oxidase (1.9 μM) was diluted in 20 mM Tris, 0.1 M NaCl, 1 mM EDTA, and 1 mg/mL lauryl maltoside at pH 7.8 and 25 °C. The enzyme was titrated with microliter amounts of 0.5 mM PDS and the reaction monitored at 434 nm. The absorbance change was completed after 10 min, and this value was used to determine the number of sulfhydryl groups reacted using an extinction coefficient of 1.98×10^4 M^{-1} cm^{-1} for the leaving group generated in the sulfide exchange reaction. The open and closed symbols are experimental results from two different days.

Gdn·HCl concentration is raised from 0 to 1 M. In 1 M Gdn·HCl, the maximum is at 422 nm, and the intensity is decreased by 30%. Upon reductant addition, the initial spectrum became more like the final spectrum as the concentration of denaturant increased. This change in the pattern of dithionite reduction indicates that dithionite can reduce both cytochrome *a* and cytochrome a_3 directly. The reduced – oxidized difference spectra are shown in Figure 3e.

The effect of Gdn·HCl concentration on the enzymic activity of cytochrome oxidase is shown in Figure 4. In this experiment, cytochrome oxidase was incubated in buffer containing different amounts of Gdn·HCl. The enzyme was then diluted 10-fold into buffer not containing Gdn·HCl and then a further 150-fold into the assay at different times following the start of the treatment. The final concentration of Gdn·HCl in the assay was at most 4 mM, a level that does not affect the activity of the untreated enzyme. The oxidase's activity was measured at 3 times following the initiation of exposure to Gdn·HCl: (1) immediately (i.e., ~30 s after Gdn·HCl exposure); (2) after 1 h; and (3) after 24 h. When assayed immediately after the addition of Gdn·HCl, 0.025 M Gdn·HCl caused a small stimulation of activity whereas higher concentrations caused inactivation. With this short time of exposure to denaturant, 3 M Gdn·HCl is required to completely inactivate the enzyme. The curve is shifted to lower Gdn·HCl concentration as the time of exposure to the denaturant is increased. After a 24-h exposure to Gdn·HCl, the oxidase activity is lost completely at 1 M denaturant concentration. Thus, the oxidase's activity is lost in the first stage of denaturation during which substantial, although incomplete, disruption of the enzyme's structure has occurred.

Cytochrome oxidase contains 17 cysteine sulfhydryl residues, 4 of which are involved in disulfide bonds, leaving, presumably, 13 potentially reactive cysteine groups. When cytochrome oxidase is titrated with the sulfhydryl reagent 4,4′-dipyridyl disulfide, only one to two sulfhydryl groups are found to be highly reactive (see Figure 5). When exposed to the denaturant Gdn·HCl, the number of reactive sulfhydryl groups increases significantly and reaches a maximum value of between 8 and 10 at 2 M Gdn·HCl. Above 2 M Gdn·HCl, the number of reactive sulfhydryls first decreases and then increases again between 4 and 7 M Gdn·HCl. At the higher Gdn·HCl concentration, there was variability between experiments due to protein aggregation. Once again, exposure of the buried sulfhydryls occurs during the first stage of denaturation.

In order to assess the structural state of the oxidase at different denaturant concentrations, we have performed gel filtration experiments on S-200 columns equilibrated at different concentrations of Gdn·HCl. The subunit content of the fractions from this column was determined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. In the absence of denaturant, we observe a single protein peak (i.e., A_{280nm}) and a single heme peak (A_{420nm}) that coeluted just after the void volume of the column (not shown). Samples of oxidase exposed to low, medium, and high concentrations of Gdn·HCl were eluted on columns equilibrated in the same low, medium, and high concentration of denaturant. The absorbance profiles were recorded at 280 and 420 nm and are shown in Figure 6. At 0.5 M Gdn HCl, the heme coeluted with a single protein peak that eluted at the same position as the native enzyme. A second peak absorbing at 280 nm, but not at 420 nm, emerges from the column at fraction 30. The "colorless" protein in fraction 30 consists of the oxidase subunit VII (see Figure 7a). The main protein peak (fraction 16) contains the oxidase subunits I, II, IV, V, VIa, VII, and VIII. Subunits III, VIb, and VIc are not recovered in this experiment and probably are irreversibly bound to the S-200 matrix. At 2.5 M Gdn·HCl, there is a substantial amount of protein emerging from the column at the position found for the native enzyme, but this peak is nearly devoid of heme (see Figure 6b). The heme emerges at fraction 20 with protein that elutes as a shoulder to the main protein peak. There are also a set of protein peaks eluting from the column between fractions 24 and 30. Electrophoretic analysis of a selection of these fractions reveals that the first protein peak (i.e., fraction 16) contains subunits I, II, IV, VII, and VIII. The protein emerging with the heme at fraction 20 is largely made up of subunits II and IV and some subunit I. Subunit V is in fraction 24, subunit VII is in fraction 27, and a mixture of subunits

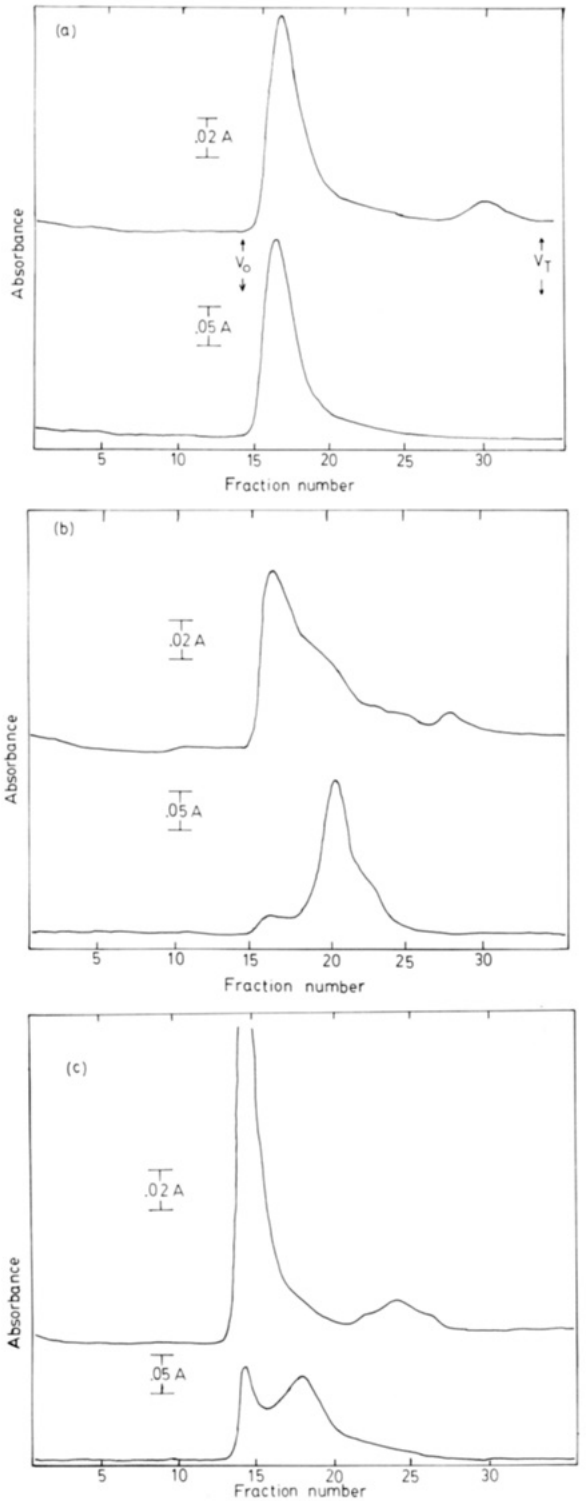

FIGURE 6: Gel filtration chromatography on Sephacryl S-200 of cytochrome oxidase at different concentrations of Gdn·HCl. The concentrations of Gdn·HCl were (a) 0.5, (b) 2.5, and (c) 5.1 M. The concentration of cytochrome oxidase was 2.15 mg/mL in buffer with the appropriate concentration of Gdn·HCl. A 0.5-mL volume of sample was applied in each experiment, and 1-mL fractions were collected. The upper trace in each panel is the A at 280 nm, and the lower trace is the A at 420 nm. The void volume (V_0) and total volume (V_T) are indicated on panel a.

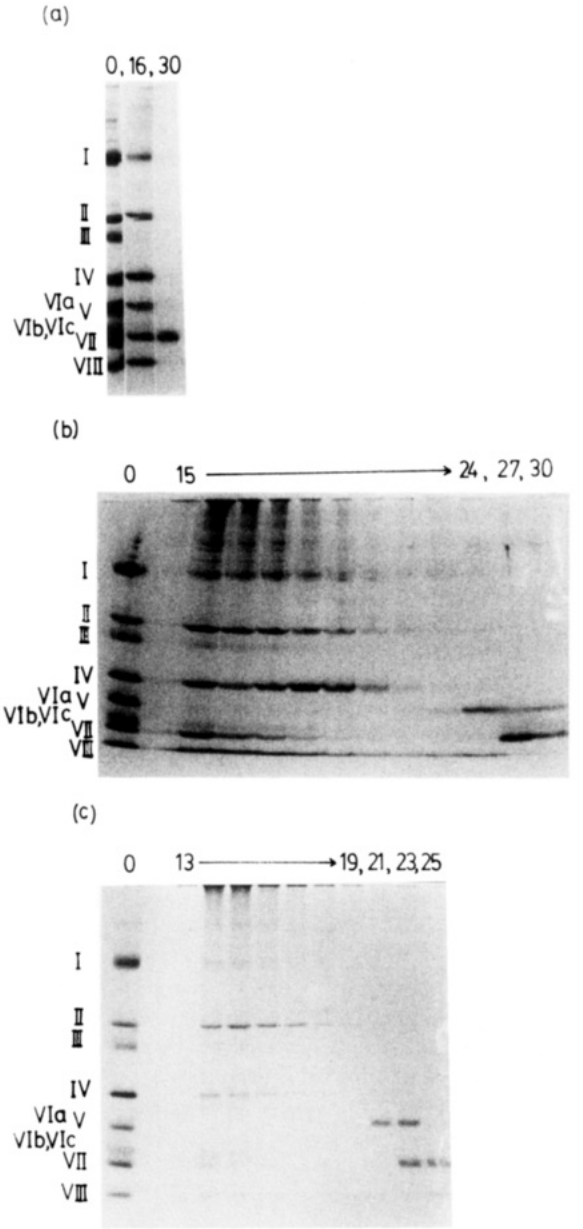

FIGURE 7: Subunit analysis of cytochrome oxidase fractionated by gel filtration chromatography in different concentrations of Gdn·HCl. Selected fractions from the experiments shown in Figure 5 were analyzed for their subunit content by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. The protein was visualized by staining with Coomassie blue. Panels a, b, and c correspond to fractions derived from cytochrome oxidase run on Sephacryl S-200 columns in 0.5, 2.5, and 5.1 M Gdn·HCl, respectively. Standard cytochrome oxidase samples are labeled as lane 0 in each gel. The subunits of the oxidase are designed along the right-hand margin according to Buse et al. (1985). The other lanes are numbered to correspond to the fractions in the elution profiles in Figure 6.

V and VII is in fraction 30. In 5 M Gdn·HCl, the majority of the protein elutes at the void volume of the S-200 column in a large aggregate with some of the heme. This major protein peak is not disrupted by SDS, and so when electrophoresed in SDS, it does not enter the polyacrylamide gel. There was also a broad peak of protein centered at fraction 23. Fraction 21 was found to consist of subunit V, fraction 23 was a mixture of subunits V and VII, and fraction 25 was a mixture of subunits VII and VIII.

DISCUSSION

The biphasic response of cytochrome oxidase to the denaturant Gdn·HCl can be viewed as a combination of the responses seen for water-soluble and integral membrane proteins. Water-soluble proteins typically unfold in a cooperative process that gives rise to a very sharp transition when they go from the native state to the denatured state. Examples of proteins of this class include lysozyme (Tanford, 1968), the polar heme-containing domain of cytochrome b_5 (Visser et al., 1975),

myoglobin (Ahmad, 1985), thioredoxin (Kelly & Stellwagon, 1984), and cytochromes of the *c* type (Hon-ami & Oshima, 1979). In contrast, the response of integral membrane proteins is quite different. They exhibit a high resistance to denaturation and unfold over a broad range of denaturant concentration (Nozaki et al., 1978; Rizzolo & Tanford, 1978; Byers & Verpoorte, 1978; Cockle et al., 1978). Cytochrome b_5 represents the case of a single polypeptide with both integral membrane and solvent-exposed domains. This protein exhibits a biphasic response to Gdn·HCl (Tajima et al., 1976). The more sensitive response is attributed to unfolding of the solvent-exposed, heme-containing domain, whereas the response at high Gdn·HCl concentration is assigned to the membrane-embedded domain.

Low-resolution structural models of cytochrome oxidase recognize two integral membrane domains (i.e., M_1 and M_2) and a solvent-exposed, cytoplasmic domain (i.e., C). The M_1 and M_2 domains span the membrane, while the C domain extends 30 Å above the surface of the bilayer. We suggest that our results on the denaturation of cytochrome oxidase by Gdn·HCl reflect this domain structure. The response of the complex to low Gdn·HCl concentration, i.e., less than 1–2 M, resembles that seen with many simple water-soluble proteins or with solvent-exposed domains of integral membrane proteins, and we assign it to the denaturation of the C domain. In contrast, the second stage in the denaturation of cytochrome oxidase by Gdn·HCl is similar to that seen for hydrophobic, integral membrane proteins, and we assign it to the denaturation of the M_1 and M_2 domains. Denaturation of the C domain is accompanied by loss of the Soret CD heme signal. Myer (1985) has resolved the Soret CD signal of cytochrome oxidase into its component contributions from the hemes of cytochrome *a* and cytochrome a_3. In our studies, this signal decreases uniformly across the Soret spectral region as denaturation proceeds and separate responses from cytochrome *a* and cytochrome a_3 are not resolved. Thus, we conclude that the environment of both heme groups is affected in the first stage of denaturation by low concentrations of Gdn·HCl, and we propose a C domain location for both hemes in the native enzyme. However, we cannot rule out the possibility that changes in the C domain could be transmitted to either of the M domains, but for now we accept the simpler explanation.

The study of the denaturation of cytochrome oxidase is complicated by the fact that the enzyme is a multisubunit complex. The response to Gdn·HCl appears to involve subunit dissociation and protein unfolding. The study of the denaturation of oligomeric proteins is not far advanced, but there is expected to be some balance between the forces holding such complexes together and the forces which establish secondary structures. At least in the case of cytochrome oxidase, this appears to be different for different subunits. Therefore, subunits such as III and VII are removed from the complex at low denaturant concentration when large perturbations in the overall secondary structure of the protein have not yet occurred.

There is strong evidence to believe that both hemes of cytochrome oxidase are bound to subunits I and II (Capaldi et al., 1983; Wikström et al., 1985). This view is based on the isolation of a complex of these components under controlled denaturation conditions (Winter et al., 1980) and by analogy with the two-subunit preparation of a cytochrome aa_3 type oxidase from *Paracoccus denitrificans* (Ludwig & Schatz, 1980). The isolation from the beef heart enzyme of a complex containing heme together with subunits I, II, and IV using Gdn·HCl (see Figures 6 and 7) and using urea (Freedman et al., 1979) we believe supports the above proposal. The presence of subunit IV in these heme complexes is probably fortuitous since it is known to be largely located on the matrix side of the oxidase complex (Fuller et al., 1985). In addition, Chance and Powers (1985) have summarized spectroscopic evidence that identifies subunit II as the site of binding of Cu_B with all the other metal centers on the cytoplasmic side of the complex. Our conclusion that both hemes are located in the C domain supports this earlier proposal.

Subunit III, which is proposed to contribute largely to one of the membrane-embedded domains of the oxidase complex (Capaldi et al., 1983), behaves anomalously in our experiments. It is not recovered from the gel filtration columns even at low levels of denaturant. This observation indicates that subunit III dissociates from the complex and either precipitates or becomes irreversibly adsorbed to the column matrix. Subunit III dissociation at low Gdn·HCl concentration is consistent with its removal by nondenaturing detergents (Azzi et al., 1980; Hill & Robinson, 1986) and its early denaturation in thermal studies (Rigell et al., 1985). Subunit III may then represent one of those proteins whose association with its functional complex is weak relative to the overall stability of the enzyme complex.

The second phase of the denaturation of cytochrome oxidase by Gdn·HCl occurs over a broad range of denaturant concentration, from 3 to 7 M Gdn·HCl. During this second stage in the denaturation of cytochrome oxidase, further protein unfolding occurs, but it is not complete even in 7 M Gdn·HCl. Cytochrome oxidase appears to retain a significant amount of β structure in 7 M Gdn·HCl as evidenced by its UV CD spectrum having a trough at 217 nm (Myer, 1985). The further unfolding that occurs in this stage gives rise to protein aggregation between some of the subunits of the complex. This aggregation probably involves hydrophobic interactions between partially unfolded subunits of the M_1 and M_2 domains. We were unable to analyze the subunit content of the aggregate as it was not dissociated in SDS. Similar behavior has also been observed in denaturation studies of the very hydrophobic coat protein of the Fd bacteriophage (Nozaki et al., 1978).

The response of the cytochrome oxidase complex to denaturation by Gdn·HCl proceeds as a two-stage process, reflecting the hydrophilic and membrane-embedded domains of the complex. The heme groups appear to be located in the Gdn·HCl-sensitive, hydrophilic C domain of the complex, which we suggest is at least partially composed of subunits I and II. Treatment of such oligomeric, multidomain proteins with low levels of denaturants may be a useful way to remove specific subunits prior to any drastic unfolding of the rest of the complex.

Registry No. Gdn·HCl, 50-01-1; EC 1.9.3.1, 9001-16-5.

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Study of the Specific Heme Orientation in Reconstituted Hemoglobins†

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Received December 8, 1987; Revised Manuscript Received February 11, 1988

ABSTRACT: NMR studies of the recombination reaction of apohemoglobin derivatives with natural and unnatural hemes and of the heme-exchange reaction for reconstituted hemoglobin have revealed that the heme is incorporated into the apoprotein with stereospecific heme orientations dependent upon the heme peripheral 2,4-substituents and the axial iron ligand(s). Heme orientations also depend on whether recombination occurs at the α or β subunit and on whether or not the complementary subunit is occupied by the heme. In the recombination reaction with the azido complex of deuterohemin, the α subunit of the apohemoglobin preferentially combines with the hemin in the "disordered" heme orientation, whereas protohemin is inserted in either of two heme orientations. Mesohemin inserts predominantly in the "native" heme orientation. For the β subunit, specific heme orientation was also encountered, but the specificity was somewhat different from that of the α subunit. It was also shown that the specific heme orientation in both subunits is substantially affected by the axial heme ligands. These findings imply that apohemoglobin senses the steric bulkiness of both the porphyrin 2,4-substituents and the axial iron ligands in the heme–apoprotein recombination reaction. To gain an insight into the effect of the protein structure, the heme reconstitution reaction of semihemoglobin, demonstrating that the heme orientation in the reconstituted semihemoglobin with the azido–deuterohemin complex was in the native form, was also examined. Moreover, when protoheme is added to a solution of the deuteroheme-reconstituted hemoglobin so that the heme-exchange reaction takes place, the resulting heme orientation for the α subunit is also quite specific (native heme orientation only), which is different from the case of the heme–apoprotein recombination reaction. Therefore, it is concluded that the orientation of the heme inserted into the apoprotein of the hemoglobin subunit depends upon the structure of the complementary subunit. In other words, the subunit structural changes induced by the tertiary structural changes of the complementary subunit through the subunit–subunit interaction subtly affect the stereospecific heme orientation.

The apparently simple and instantaneous (~1 ms) reconstitution reaction in vitro of heme and apohemoglobin (apoHb)[1] to yield unique holoproteins (Gibson & Antonini, 1960; rose & Olson, 1983) leads to the view that the last step of the hemoprotein biosynthesis is a rapid folding process which affords the same species as found in single crystals (Gibson & Antonini, 1960; Adams, 1976, 1977). Recent ^{1}H NMR studies have demonstrated that the reconstitution of heme with apoMb and apoHb proceeds via a random isomeric incorporation, with respect to rotation about the α,γ-meso axis of

† This work was supported by grants from the Ministry of Education Japan (62220018 and 62790145).
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[1] Abbreviations: NMR, nuclear magnetic resonance; Hb A, human adult hemoglobin; metHb, ferric hemoglobin; Mb, myoglobin; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; ppm, parts per million; MEK, methyl ethyl ketone; WEFT, water-eliminated Fourier transform; semiHb, semihemoglobin; apoHb, apohemoglobin; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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