Proteolysis of the Cytochrome *d* Complex with Trypsin and Chymotrypsin Localizes a Quinol Oxidase Domain†

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ABSTRACT: The cytochrome *d* complex is a two-subunit, membrane-bound terminal oxidase in the aerobic respiratory chain of *Escherichia coli*. The enzyme catalyzes the two-electron oxidation of ubiquinol and the four-electron reduction of oxygen to water. Previous work demonstrated that the site for ubiquinol oxidation was selectively inactivated by limited proteolysis by trypsin, which cleaves at a locus within subunit I. This work is extended to show that a similar phenomenon is observed with limited chymotrypsin proteolysis of the complex. The cleavage patterns are similar whether one uses the purified oxidase in nondenaturing detergent or reconstituted in proteoliposomes or uses spheroplasts of *E. coli* as the substrate for the proteolysis. Hence, the protease-sensitive locus is periplasmic in the cell. Fragments resulting from proteolysis were characterized by N-terminal sequencing and by immunoblotting with the use of a monoclonal antibody of known epitope within subunit I. The data indicate that inactivation of the ubiquinol oxidase activity results from cleavage at specific residues with a hydrophilic region previously defined as the Q loop. This domain has been already implicated in ubiquinol oxidation by the use of inhibitory monoclonal antibodies. Electrochemical and HPLC analysis of the protease-cleaved oxidase suggests no global changes in either the quaternary or tertiary structure of the enzyme. It is likely that the Q loop is directly involved in forming a portion of the ubiquinol binding site near the periplasmic surface of the membrane.

The cytochrome *d* complex (or cytochrome *bd*) is one of two terminal oxidases of the aerobic respiratory chain of *Escherichia coli* (Anraku & Gennis, 1987; Anraku, 1988). It catalyzes the oxidation of ubiquinol 8 within the cytoplasmic membrane (Koland et al., 1984a) and the reduction of molecular oxygen to water (Minghetti & Gennis, 1988). Turnover of the oxidase results in the generation of a proton electrochemical gradient across the membrane (Koland et al., 1984; Miller & Gennis, 1985); i.e., this enzyme is a coupling site for energy conservation. When the oxygen tension in the growth medium is low, synthesis of the enzyme is enhanced and the oxidase accumulates in the membrane (Rice & Hempfling, 1978; Kranz & Gennis, 1984; Iuchi et al., 1990; Cotter et al., 1991; Georgiou et al., 1988b). The affinity of the cytochrome *d* complex for oxygen is significantly higher than that of the cytochrome *o* complex, which is the predominant terminal oxidase when *E. coli* is grown under conditions with high aeration (Rice & Hempfling, 1978; Smith et al., 1990). The role of the *d*-type oxidase appears to be 2-fold (Hill et al., 1990): (1) energy conservation under microaerobic conditions and (2) protection of sensitive anaerobic enzymes from molecular oxygen.

The cytochrome *d* complex has been purified (Miller & Gennis, 1983; Kita et al., 1984) and reconstituted in proteoliposomes (Koland et al., 1984a; Miller & Gennis, 1985). The oxidase contains one copy each of two subunits (Miller et al., 1988), and both subunits are encoded by the *cyd* operon, which has been cloned (Green et al., 1984) and sequenced (Green et al., 1988). There are three heme prosthetic groups (Meinhardt et al., 1989; Lorence et al., 1988). Cytochrome b_{558} is located within subunit I (58 000 Da) (Green et al., 1986), whereas cytochromes b_{595} and *d* require both subunits to bind. Subunit I has been implicated as being directly involved in the oxidation of ubiquinol, presumably via cytochrome b_{558}. This was initially indicated by the binding of a ubiquinol photoaffinity probe (Yang et al., 1986). Also, monoclonal antibodies that bind subunit I specifically inhibit ubiquinol oxidation but do not inhibit the oxidation of an artificial reductant, TMPD[1] (Dueweke & Gennis, 1990). The epitope for these monoclonal antibodies has been localized to within an 11 amino acid stretch in a large hydrophilic loop (Q loop) between the fifth and sixth transmembrane spans of subunit I (Dueweke & Gennis, 1990). Furthermore, this Q loop has been demonstrated to be on the periplasmic side of the *E. coli* inner membrane (Dueweke & Gennis, 1990).

Previous studies have demonstrated that trypsin proteolysis of the purified cytochrome *d* complex selectively cleaves subunit I, resulting in the loss of ubiquinol oxidase activity without inhibition of the TMPD oxidase activity (Lorence et al., 1988). The purpose of the current work is to further characterize this proteolytic inactivation. The results show that both trypsin and chymotrypsin cleave subunit I of the oxidase at sites located within the Q loop very close to the epitope for the inhibitory monoclonal antibodies. Proteolysis of spheroplasts confirms the periplasmic localization of this domain. The cleaved, purified oxidase shows no alteration in the electrochemical properties of the heme prosthetic groups and no gross change in quaternary structure. The data suggest that the inactivation is probably due to the loss of ability of the cytochrome *d* complex to bind to ubiquinol. Possibly, the

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[1] Abbreviations: TMPD, *N,N,N′,N′*-tetramethylphenylenediamine; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; PVDF, poly(vinylidine difluoride); TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; TLCK, *N*-α-tosyl-L-lysine chloromethyl ketone.

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Q loop forms part of the binding site for ubiquinol.

Materials and Methods

Bacterial Strains. The *E. coli* strain GR84N carrying the plasmid pNG2, which contains the *cyd* operon and is used to overexpress the cytochrome *d* complex, has been described previously (Green et al., 1984). The plasmid confers tetracycline resistance.

Materials. TLCK-treated trypsin, TPCK-treated chymotrypsin, phenazine methosulfate, and potassium ferricyanide were purchased from Sigma. Aldrich Chemical Co. supplied 2-hydroxy-1,4-naphthoquinone. Quinhydrone, 1,2-naphthoquinone, anthraquinone-2-sulfonate, and sodium dithionite were obtained from Eastman Kodak Co.

Preparation of GR84N/pNG2 Spheroplasts. A sample of 40 mL of an overnight culture of GR84N/pNG2, grown in LB medium plus 12.5 μg/mL tetracycline, was pelleted and washed once in 30 mM Tris-HCl, pH 8.0. The resulting pellet was resuspended in 4 mL of buffer containing 20% sucrose, 30 mM Tris-HCl, and 2 mM PMSF, pH 8.0. EDTA, pH 8.0, was added to a final concentration of 10 mM followed by the addition of lysozyme (1 μg/mL). The suspension was incubated on ice with occasional swirling for 30 min. $MgSO_4$ was added to a final concentration of 20 mM followed by addition of DNase I (10 μg/mL). After an additional incubation of 10 min on ice, the spheroplasts were pelleted by centrifugation for 10 min at 10000*g*. The pellet was resuspended in 4 mL of 20% sucrose/30 mM Tris-HCl/20 mM $MgSO_4$/10 μg/mL DNase I, pH 8.0. This, without the DNase, is referred to as spheroplast buffer. After another two pelleting and resuspension steps with spheroplast buffer, an aliquot was removed for quantitation of the cytochrome *d* complex by measuring the reduced-*minus*-oxidized absorbance spectrum (Lorence et al., 1986; Georgiou et al., 1988a). The concentration was typically around 100 μg of cytochrome *d* complex/mL of spheroplast preparation.

Proteolysis, SDS-PAGE, and Immunoblotting of Spheroplasts. A total of 5 μL of spheroplasts containing 20 μg/mL of cytochrome *d* complex was incubated for 1 h at 25 °C with 1 μL of the protease solution of the desired dilution. Proteolysis was stopped by the addition of PMSF to a final concentration of 2 mM. All dilutions were performed with spheroplast buffer. The samples were subjected to electrophoresis using an SDS-PAGE system with urea (Lorence et al, 1986). The protein bands were transferred to nitrocellulose for Western immunoblotting (Burnette, 1981) and probed with a mixture of polyclonal antibodies monospecific for subunit I or II of the cytochrome *d* complex.

Preparation of Proteoliposomes. Proteoliposomes were prepared by a detergent dilution method. A 750-μL sample of 25 mg/mL phosphatidylethanolamine was mixed with 250 μL of 25 mg/mL phosphatidylglycerol and dried under a stream of N_2. Last traces of the organic solvent (chloroform) were removed by vacuum for 2 h, and the lipids were resuspended in 4 mL of 1.25% octyl glucoside/50 mM potassium phosphate, pH 7.5, at 25 °C. A total of 200 μL of a solution containing the purified cytochrome *d* complex (2.7 mg) in 0.05% Sarkosyl/600 mM NaCl/10 mM Tris-HCl, pH 8.2, was added, and the mixture was incubated on ice for 20 min with occasional swirling. The detergent-solubilized protein–lipid mixture was then added to 138 mL of 50 mM potassium phosphate, pH 7.5, at 25 °C and then stirred for an additional 10 min. The solution was then centrifuged for 1 h at 45 000 rpm in a Beckman 60 Ti rotor. The pellets were resuspended in a total of 750 μL of 50 mM Tris-HCl, pH 7.5, and subjected to brief sonication, 30–40 pulses of 50% cycle at a power setting of 2 on a Branson W-250 sonicator with a 1-cm-diameter probe, until the solution became opalescent. Proteolysis was achieved by incubation of the proteoliposomes with either trypsin or chymotropsin at a concentration of 1/1000 (w/w) relative to the amount of cytochrome *d* for 30 min at 25 °C. Proteolysis was stopped by the addition of PMSF to a final concentration of 2 mM.

Proteolysis of the Purified, Detergent-Solubilized Cytochrome d Complex. All digestions of the purified complex were performed at 25 °C with both protein and protease in 0.05% Sarkosyl/600 mM NaCl/10 mM Tris-HCl, pH 8.2. Protease concentrations expressed in the text are given as weight to weight ratios relative to the amount of cytochrome *d* complex. Reactions were stopped by addition of PMSF to a final concentration of 2 mM.

Kinetics of Proteolytic Inactivation. Protease (0.2 μg/mL) was added to 2.5 mg/mL cytochrome *d* complex in the buffer containing 0.05% Sarkosyl, 600 mM NaCl, and 10 mM Tris-HCl, pH 8.2. Aliquots were withdrawn at a series of times and diluted 40-fold in the same buffer plus 2 mM PMSF to stop proteolysis. A 4-μL sample of each diluted aliquot, containing 250 ng of cytochrome *d* complex, was added to 1.8 mL of 0.05% Triton X-100/1 mM EDTA/2 mM dithiothreitol/25 mM Tris-HCl, pH 7.5, and equilibrated to 37 °C in a temperature-controlled, stirred vessel fitted with an oxygen electrode (YSI Model 53). After a few seconds, a flat base line was observed and ubiquinol-1 was added to 250 μM to observe quinol oxidase activity. To assess TMPD oxidase activity, 1 mM TMPD was added first in order to observe the extent of background activity. The cytochrome *d* aliquot was then added, and the net oxidase activity was determined.

Densitometry Traces of SDS-PAGE Gels. The density of Coomassie-stained protein bands in SDS-PAGE gels was determined by use of an LKB Ultroscan XL laser densitometer.

Transfer of Proteins from SDS-PAGE Gels to Poly(vinylidine difluoride) Membrane for Direct Microsequencing of the Amino termini. The protein bands resulted from proteolysis of the cytochrome *d* complex by trypsin or chymotropsin were transferred electrophoretically from SDS-PAGE gels to poly(vinylidine difluoride) (PVDF) membranes (Matsudaira, 1987; Hunkapiller et al., 1986; Aebersold et al., 1986). For each protease, one sample of cytochrome *d* complex was digested for 1 h with 1/10 000 (w/w) and another was digested for 4 h with 1/2500 (w/w) of the protease. A 250-μg sample of protein was loaded per lane from each digestion for resolution by SDS-PAGE. Following transfer to PVDF, the bands of interest from each protease digestion were located, excised, and submitted for N-terminal amino acid sequencing by the Genetic Engineering Faculty of the University of Illinois.

Potentiometric Analysis of Native and Trypsin-Cleaved Cytochrome d Complex. Spectrophotometric titrations of the heme components in native and trypsin-cleaved cytochrome *d* complex were performed by poising the redox potential chemically (Lorence et al., 1984). The buffer used was 0.05% Triton X-100/1 mM EDTA/25 mM Tris-HCl, pH 7.5, with the following redox mediators added to a 5 mM final concentration: quinhydrone (midpoint potential at pH 7.0 (E_m) = +280 mV), 1,2-naphthoquinone-4-sulfonate (E_m = +215 mV), 1,2-naphthoquinone (E_m = +143 mV), phenazine methosulfate (E_m = +10 mV), 2-hydroxy-1,4-naphthoquinone (E_m = −145 mV), and anthraquinone-2-sulfonate (E_m = −225 mV). To this buffer was added 400 μL of 5.1 μg/mL cytochrome *d* complex in 0.05% Triton X-100/1 mM EDTA/25

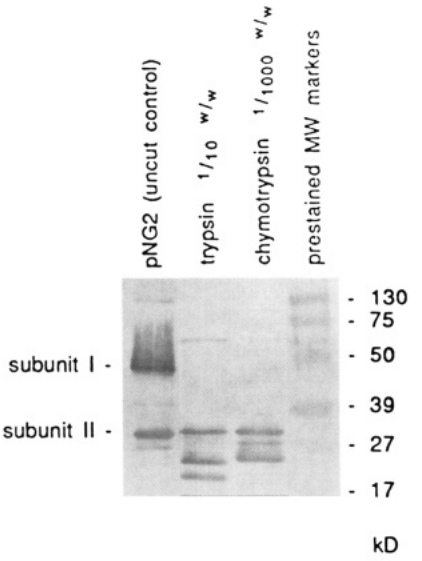

FIGURE 1: Western blot showing the effects of proteolysis of spheroplasts expressing the cytochrome *d* complex. Spheroplasts from a strain that overproduces the cytochrome *d* complex (GR84N/pNG2) were subjected to proteolysis with trypsin or chymotrypsin at the indicated concentrations (relative to cytochrome *d* content). Polypeptides were visualized by use of a mixture of polyclonal antibodies monospecific for both subunit I and subunit II of the cytochrome *d* complex. Prestained molecular weight markers: phosphorylase *b*, 130000; bovine serum albumin, 75000; ovalbumin, 50000; carbonic anhydrase, 39000; soybean trypsin inhibitor, 27000; lysozyme, 17000. Subunit-specific antisera show no evidence for cleavage of subunit II (not shown).

mM Tris-HCl, pH 7.5, and oxygen-free argon was passed over the solution in the stirred cell under slight positive pressure for 2 h to remove oxygen. During this time, the solution electrochemical potential typically fell from 330 to 280 mV and a gradual loss of the peak amplitude at 650 nm in the oxidized spectrum was observed. This absorbance peak is indicative of oxygen binding to the cytochrome *d* complex (Koland et al., 1984b; Lorence & Gennis, 1989). The solution potential was then raised to +380 mV by the addition of successive aliquots of deoxygenated 5 mM potassium ferricyanide (E_m = +430 mV), and a base-line, "oxidized" spectrum was taken scanning from 500 to 700 nm. Successive spectra were then recorded as the solution potential was gradually lowered via addition of small aliquots of freshly made, deoxygenated 10 mM sodium dithionite. Both the ferricyanide and dithionite solutions were prepared in buffer containing 0.05% Triton X-100, 1 mM EDTA, and 25 mM Tris-HCl, pH 7.5. For the potentiometric titration of trypsin-cleaved cytochrome *d* complex, 400 μL of 5.1 mg/mL complex was proteolyzed with 1/1000 (w/w) trypsin for 30 min at 25 °C and PMSF was added to a final concentration of 2 mM. SDS–PAGE analysis was performed on aliquots taken after the potentiometric titrations to confirm the cleaved or native state of the cytochrome *d* complex.

The spectra were obtained as hard copy by use of a Varian-Cary 219 spectrophotometer. Absorbance values at 628, 571, and 538 nm were used to determine the fractional reduction of cytochromes *d*, b_{595}, and b_{558}, respectively. All absorbance values for a given heme species were then plotted as percentages of the highest absorbance values observed for that heme, i.e., the most reduced state.

Analytical HPLC Gel Filtration Chromatography. The quaternary structure of the native, trypsin-cleaved, and chymotropsin-cleaved forms of the oxidase were compared by analytical gel filtration chromatography using a Beckman TK658 spherogel column. The buffer used was 0.05% Sarkosyl/600 mM NaCl/10 mM Tris-HCl, pH 8.2. Approximately 6 μg of protein was loaded in 10 μL, and the elution profile was monitored by the absorbance at 280 nm.

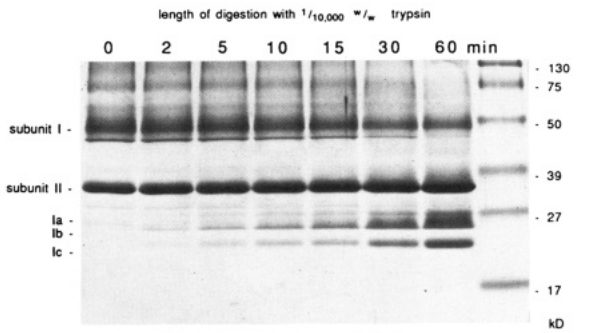

FIGURE 2: Kinetics of the proteolysis of the purified, detergent-solubilized cytochrome *d* complex with trypsin. The cytochrome *d* complex was subjected to proteolysis with trypsin at a concentration of 1/10000 (w/w) relative to the amount of cytochrome *d*. The time following the addition of the trypsin is indicated above each lane. Proteolytic fragments of subunit I are labeled Ia, Ib, and Ic.

RESULTS

Figure 1 is a Western immunoblot that shows the results of proteolysis by trypsin or chymotrypsin of spheroplasts containing the cytochrome *d* complex. It is evident in both cases that subunit I is vulnerable to proteolysis whereas subunit II is apparently not affected. In the case of trypsin, two major proteolytic products are evident, with several far less abundant bands migrating between subunit II and the major fragments. Proteolysis of spheroplasts with chymotrypsin gives rise to what appears to be one major proteolytic product band, with associated minor bands similar to those observed with trypsin proteolysis. In both cases, the proteolysis indicates that there are proteolytically sensitive sites within subunit I that are directed towards the periplasm.

Similar results were obtained by use of proteoliposomes containing the pure cytochrome *d* complex (not shown). Previous studies using trypsin proteolysis demonstrated that the cytochrome *d* complex is oriented about 70–80% right-side-out in proteoliposomes (Lorence et al., 1988). About 20–30% of subunit I is protected from externally added proteases, unless a detergent is present that disrupts the bilayer. Presumably, this reflects a subpopulation of the complex in which the protease-sensitive sites are located inside the closed vesicles. There is no indication of new sites revealed in the subpopulation that are oriented inside-out. The proteolytic fragment pattern observed with proteoliposomes mirrors that observed with spheroplasts (Figure 1).

When pure, detergent-solubilized cytochrome *d* complex is subjected to proteolysis with trypsin or chymotropsin, the patterns of fragment bands observed by SDS–PAGE are also similar to those observed with spheroplasts. Figure 2 shows the time course of digestions of pure cytochrome *d* complex with trypsin. The improved resolution gained by use of pure protein as a substrate reveals that three distinct product bands actually result. These are labeled fragments Ia, Ib, and Ic (Figure 2). Reference to the digests of spheroplasts and proteoliposomes suggests that fragment Ia is also present in these cases but is poorly resolved from fragment Ib. Chymotrypsin digestion of pure cytochrome *d* complex shows two rapidly appearing product bands, Ia and Ib, and a third band, Ic, that appears only after prolonged proteolysis (not shown). Bands Ia and Ib are also not resolved where proteoliposomes are used as a substrate. Clearly, in a detergent-soluble form as well as in membrane systems, the cytochrome *d* complex has a domain in subunit I that is dramatically more sensitive

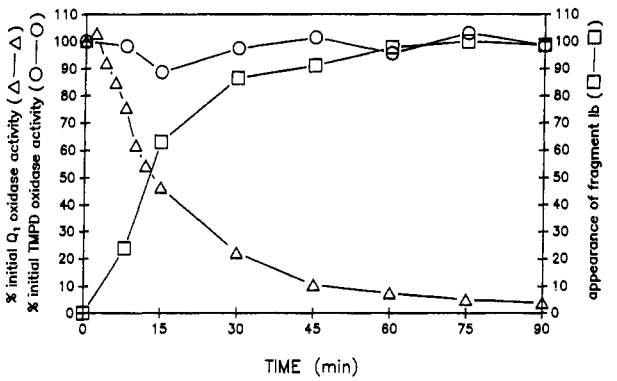

FIGURE 3: Kinetics of trypsin digestion of the purified cytochrome *d* complex in detergent: ubiquinol 1 oxidase activity, TMPD oxidase activity, and appearance of proteolytic products as a function of the time of proteolysis. Aliquots following the addition of 1/12500 (w/w) trypsin were assayed for ubiquinol 1 oxidase activity (Δ) and TMPD oxidase activity (O) and subjected to SDS–PAGE. The appearance of the proteolytic fragment Ib is plotted as a percentage of the amount observed after 90 min (□) and was quantified densitometrically. Specific activities observed for the native cytochrome *d* complex not exposed to protease were 7900 mol of O_2 min^{-1} mol of cytochrome *d* with ubiquinol 1 as substrate and 1300 mol of O_2 min^{-1} mol of cytochrome *d* with TMPD as substrate.

to proteolysis with trypsin or chymotrypsin than is the rest of the complex. The results obtained with spheroplasts show that this domain is directed toward the periplasm.

The kinetics of the trypsin proteolysis is shown in Figures 2 and 3. The ubiquinol oxidase activity, measured with use of ubiquinol 1, a water soluble analogue of the in vivo substrate, ubiquinol 8, drops rapidly upon proteolysis with either trypsin (Figure 3) or chymotrypsin (not shown) in parallel with the cleavage apparent by SDS–PAGE analysis. As reported previously with trypsin (Lorence et al., 1988), the specific oxidase activity of the complex using TMPD, an artificial electron donor, in place of ubiquinol is unaffected by proteolysis. Hence, the damage resulting from proteolysis by trypsin or chymotrypsin is specific for the site of quinol oxidation.

To localize the proteolytic cleavage sites within subunit I, the three fragment bands resulting from proteolysis with trypsin and the first two resulting from proteolysis with chymotrypsin were transferred to a PVDF membrane and used for N-terminal amino acid sequencing. The chymotryptic fragment Ic appears only after extensive proteolysis, well after the specific inhibition of quinol oxidase activity is observed, and was not a subject of further investigation. For trypsin proteolysis, only fragment Ib yielded sequence data, with the cleavage site shown in Figure 4 deduced from 20 cycles of sequence. This cleavage site is located close to and to the C-terminal side of the epitope defining the binding site for monoclonal antibodies (see Figure 4) that specifically inhibit the ubiquinol oxidase activity of the enzyme (Dueweke & Gennis, 1990). This is within the periplasmic Q loop (Dueweke & Gennis, 1990). A Western blot (not shown) shows that this epitope is present only in fragment Ia, which appears only transiently. A plausible hypothesis is that trypsin cleavage at the site is shown in Figure 4 produces fragments Ia and Ib, resulting in activation of the ubiquinol oxidase activity. Relatively rapid subsequent proteolysis of fragment Ia at a site very close to or within the antibody epitope could generate fragment Ic, which fails to bind to the monoclonal antibody. Since the N-terminus of subunit I is known to be blocked (Miller et al., 1988), this is consistent with the inability to obtain sequence data from fragments Ia and Ic.

Of the two fragments resulting from proteolysis of the cytochrome *d* complex with chymotrypsin, only fragment Ib yielded N-terminal sequence data. This chymotrypsin cleavage site is also shown in Figure 4, on the basis of 18 sequencing cycles. The failure to obtain sequence data from fragment Ia can be attributed to its inclusion of the blocked N-terminus of subunit I. Consistent with this interpretation, fragment Ia from proteolysis with chymotrypsin binds to the inhibitory monoclonal antibody and contains the epitope shown in Figure 4 (not shown).

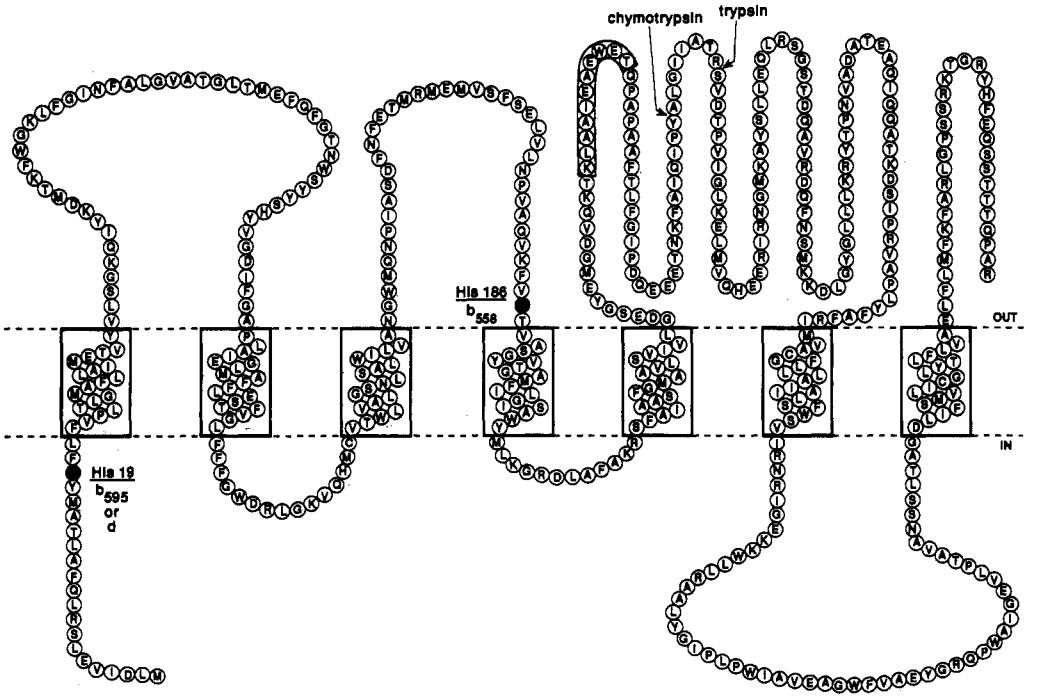

FIGURE 4: Model of subunit I of the cytochrome *d* complex showing the Q loop (Dueweke & Gennis, 1990) with proteolytic cleavage sites and the monoclonal antibody epitope (boxed region). Both proteases and the monoclonal antibodies specifically inhibit quinol oxidase activity of the cytochrome *d* complex. The probable proximity of the Q loop and His-186, an axial ligand of cytochrome b_{558} (Fang et al., 1989), suggests that the Q loop is part of a quinol oxidase domain near or including cytochrome b_{558}, a postulated acceptor of electrons from ubiquinol.

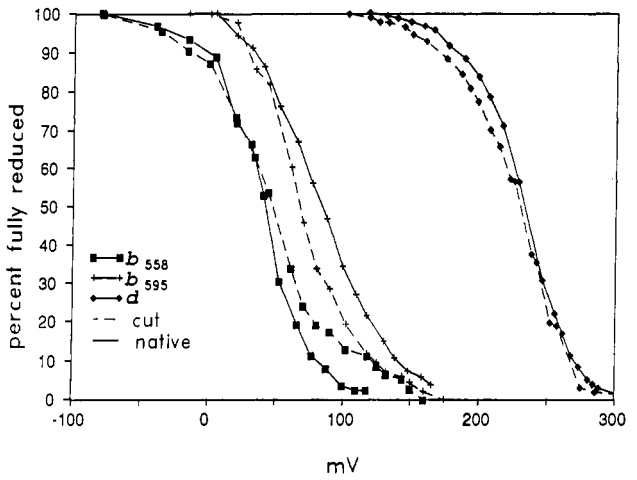

FIGURE 5: Potentiometric titrations of the heme prosthetic groups in the native and trypsin-cleaved cytochrome *d* complex. The titrations were performed as described in the text. Shown are titration profiles for the three heme components: cytochrome b_{558} (■); cytochrome b_{595} (+); and cytochrome *d* (◆). The fractional reduction of the heme components was determined by the absorbance changes at 538, 571, and 628 nm. Solid lines represent native cytochrome *d* complex; dashed lines represent trypsin-cleaved cytochrome *d* complex.

To examine the extent of the changes caused by proteolytic digestion, both the native and cleaved forms of the complex were resolved under nondenaturing conditions by use of HPLC gel filtration chromatography. Both protease-cleaved forms yield a single peak with a retention time identical with that of the uncut form, monitored by absorbance at 280 nm (not shown). In addition, the integrated area under each peak showed no significant loss of protein from the complex due to proteolysis. Hence, the quaternary structure of the enzyme appears unaffected by this limited proteolysis.

A more rigorous assessment of the effect of proteolysis on the integrity of the cytochrome *d* complex was performed by determining the effect of trypsin cleavage on the midpoint potentials of the heme groups present in the complex. Figure 5 shows that proteolysis of the complex with trypsin does not significantly affect the observed midpoint potentials of the three cytochrome components of pure cytochrome *d* complex, solubilized in 0.05% Triton X-100 at pH 7.5. The midpoint potentials observed for both the native and trypsin-cleaved complex are similar to those observed in previous work (Koland et al., 1984b).

Discussion

Previous work demonstrated that proteolysis of the purified cytochrome *d* complex by trypsin results in cleavage within subunit I and that the ubiquinol oxidase activity of the enzyme is selectively inactivated by this cleavage (Lorence et al., 1988). The present work substantially extends these studies. Several conclusions can be drawn.

(1) Proteolysis by chymotrypsin as well as by trypsin results in selective cleavage of subunit I and inactivation of the ubiquinol oxidase activity of the cytochrome *d* complex. This suggests a region or domain of the oxidase that is significantly exposed to allow access to the proteases.

(2) Essentially the same cleavage patterns are observed for detergent-solubilized enzyme, for the oxidase reconstituted in phospholipid vesicles, or for spheroplasts. Hence, there is only one protease-sensitive domain, and it faces the periplasm.

(3) The inactivation of the oxidase corresponds to proteolytic cleavage by either trypsin or chymotrypsin at unique sites that are separated by only eight amino acid residues and are within the Q loop. This Q loop has been defined previously as a hydrophilic region of subunit I that contains the epitope for monoclonal antibodies that inhibit the quinol oxidase activity of the enzyme (Dueweke & Gennis, 1990). Hence, this portion of subunit I, between the fifth and sixth transmembrane spans, has already been shown to be sufficiently exposed to bind antibodies, to be periplasmic, and to be involved somehow in ubiquinol oxidation. The localization of the protease cleavage sites in this region further emphasizes the importance of this domain. Histidine-186 is a heme ligand for cytochrome b_{558}, which is likely to be directly involved in quinol oxidation. This histidine, shown in Figure 4, must be close to the Q loop.

(4) The quaternary structure of the purified cytochrome *d* complex does not appear to be altered by proteolytic cleavage.

(5) The electrochemical behavior of the three heme prosthetic groups is also unchanged following trypsin cleavage. This suggests no global changes in the tertiary structure of the oxidase. It also demonstrates that the inability of the protease-cleaved enzyme to oxidize ubiquinol is not due to an alteration of the electrochemical properties of cytochrome b_{558}.

Altogether, these data indicate the subtle nature of the inactivation of the ubiquinol oxidase activity due the proteolytic cleavage of subunit I within the Q loop. It is possible that this portion of the protein is directly involved in forming a portion of the ubiquinol binding site near the membrane surface. This will be further examined by use of molecular genetics techniques.

Registry No. Cytochrome *d*, 9035-36-3; ubiquinol oxidase, 69671-26-7; trypsin, 9002-07-7; chymotrypsin, 9004-07-3.

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Time-Resolved Fluorescence Studies of Genetically Engineered *Escherichia coli* Glutamine Synthetase. Effects of ATP on the Tryptophan-57 Loop†

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ABSTRACT: Single-tryptophan-containing mutants of low adenylylation state *Escherichia coli* glutamine synthetase (wild type has two tryptophans at positions 57 and 158) have been constructed and studied by multifrequency phase/modulation fluorescence spectroscopy. The W57L mutant (retains tryptophan at residue 158) and the W158S mutant (retains tryptophan at residue 57) are both characterized by heterogeneous exponential decay kinetics. Global analysis indicates that for the Mn-bound form of the enzyme at pH 7.4 the fluorescence of both tryptophans is best described by a sum of three discrete exponentials with recovered lifetimes of 4.77, 1.72, and 0.10 ns for Trp-57 and 5.04, 2.28, and 0.13 ns for Trp-158. The wild-type enzyme also exhibits decay kinetics described by a triple-exponential model with similar lifetime components. The individual tryptophans are distinguishable by the fractional intensities of the resolvable lifetimes. The wild-type and W158S enzymes are dominated by the 5-ns component which provides nearly 60% and 65%, respectively, of the fractional intensity at five wavelengths spanning the emission spectrum. In contrast, the W57L enzyme demonstrates a larger fraction of the 2-ns lifetime species (60%) and only 35% of the longer lifetime component. The substrate ATP induces a shift to approximately 90% of the 5-ns component for the wild-type and W158S enzymes, whereas the W57L protein is essentially unaffected by this ligand. Steady-state quenching studies with iodide indicate that addition of ATP results in a 3.0-3.5-fold decrease in the apparent Stern-Volmer quenching constants for the wild-type and W158S enzymes. Phase/modulation experiments at several iodide concentrations indicate that the median, 2 ns, lifetime component is selectively quenched compared to the 5-ns lifetime component. These results suggest a model where ATP binding results in a shift in the equilibrium distribution of microconformational states populated by Trp-57. ATP shifts this equilibrium nearly completely to the states exhibiting the long-lifetime component which, based on quenching studies, is less solvent-accessible than the conformational states associated with the other lifetime components.

The dynamic nature of proteins, an essential aspect of their function, is exemplified by rotational motions of amino acid side chains, microconformational heterogeneity of localized peptide segments, and ligand-induced changes in domain structure and conformation (Demchenko, 1986; DeBrunner & Fraunfelder, 1982). These types of molecular motion are amenable to study by various types of fluorescence spectroscopies which sensitively reflect changes in the local environment of many fluorophores, including changes which take place on the nanosecond time scale (Alcala et al., 1987; Demchenko, 1986; Lakowicz, 1983). We have utilized phase/modulation and steady-state fluorescence techniques with site-directed mutants to determine the nature of the local environment of the individual tryptophan residues of *Escherichia coli* glut-

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