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Proximity Mapping the Surface of a Membrane Protein Using an Artificial Protease: Demonstration That the Quinone-Binding Domain of Subunit I Is near the N-Terminal Region of Subunit II of Cytochrome *bd*†

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ABSTRACT: A novel experiment has been used to show proximity relationships between sites on the surface of the cytochrome *bd* quinol oxidase of *Escherichia coli.* The artificial protease iron (*S*)-1-[*p*-(bromoacetamido)benzyl]-EDTA (Fe−BABE) was conjugated to selected reactive cysteines placed in subunit I or subunit II, with the aim of identifying amino acid residues within ≈12 Å of each site of attachment. The protease was activated with H_2O_2 and ascorbate for a few seconds, and hydrolysis products were isolated and analyzed by N-terminal sequencing. Among other results, we found that residue 39 of subunit II is near residue 255 of subunit I in the putative quinone-binding domain (Q loop) of the oxidase. Since this technique is insensitive to the nature of the amino acid side chains, it should prove generally valuable in revealing spatial relationships both within and between subunits in complex proteins where high-resolution structural information is not available.

Obtaining structural information about membrane proteins is a major experimental challenge. As demonstrated below, information on the proximity of particular amino acid residues can be gained through the use of the reagent Fe−BABE.[1] After a single reactive cysteine residue is placed in a position of interest, Fe−BABE can be attached and used to hydrolyze nearby peptide bonds. These bonds can be located within the same subunit or in a different subunit of a multisubunit complex. Their position in the primary sequence of the protein can be determined by identifying the new N-termini formed upon hydrolysis. Hence, useful constraints concerning the three-dimensional folding of the subunits and about the subunit interface regions can be derived. Since residues that are identified as neighbors by this technique must be located on the same side of the membrane, this also provides an experimental check on proposed two-dimensional models predicting which regions of each polypeptide are on each side of the membrane.

The inception and development of methods for site-specific cleavage of proteins with metal chelates have led quickly to artificial proteolytic reagents that are directed by proximity to a peptide bond, rather than by an amino acid residue type (Hoyer et al., 1990; Rana & Meares, 1990, 1991a; Schepartz & Cuenoud, 1990). This work was motivated by the use of Fe−EDTA and other chelates to study nucleic acids and their complexes, which has provided insights that would be difficult to gain otherwise (Celander & Cech, 1991; Dervan, 1992; Price & Tullius, 1992; Sigman, 1990). Most of these procedures are based on the rich chemistry of H_2O_2 (or O_2) and iron, which depends on the nature of the iron chelate involved (Sawyer, 1991; Wink et al., 1994). For oxidative scission of nucleic acids the generation of electrophilic species such as hydroxyl radicals is important (Celander & Cech, 1991; Dervan, 1992; Price & Tullius, 1992), while the hydrolysis of protein backbones by Fe−BABE appears to be dominated by nucleophilic iron−peroxo complexes (Rana & Meares, 1991b). Some applications of cysteine-linked Fe−EDTA complexes have exploited the oxidative chemistry to cut DNA (Mazzarelli et al., 1993) or RNA (Han et al., 1994; Heilek et al., 1995), and nicking of DNA by protein conjugates of copper−1,10-phenanthroline has been similarly investigated (Celander & Cech, 1991; Pan et al., 1994). There is a growing recognition of the potential value of such reagents for mapping protein sites. Recently, nonspecific cleavage of the cAMP receptor protein by untethered Fe−EDTA has been used to reveal a footprint (Heyduk & Heyduk, 1994). Cysteine-linked Fe−EDTA chelates also have been used to study non-native conformations of staphylococcal nuclease during refolding of the protein (Celander & Cech, 1991; Ermácora et al., 1992).

The chemistry of Fe−BABE has been characterized previously by using proteins of known sequence or structure (Rana & Meares, 1990, 1991a,b). Protein−Fe−BABE conjugates are formed under mild reaction conditions and are stable for long periods in the absence of activating reagents. Activation of the chelated iron is accomplished by a brief exposure (≈10 s) to H_2O_2 in the presence of

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[1] Abbreviations: Fe−BABE, iron (*S*)-1-[*p*-(bromoacetamido)benzyl]-EDTA; ATP, adenosine triphosphate; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); HAP, hydroxyapatite; PMSF, phenylmethanesulfonyl fluoride.

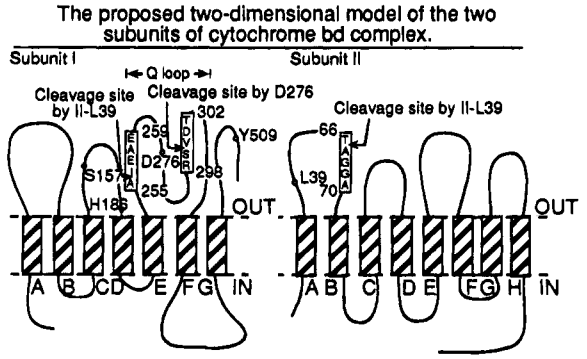

FIGURE 1: Proposed two-dimensional model of the two subunits of cytochrome *bd*. Shown are the four residues that were modified (S157, D276, and Y509 in subunit I; L39 in subunit II). The region between transmembrane spans E and F in subunit I has been implicated in quinol binding and is called the Q loop. The cleavage sites resulting from Fe−BABE adducts to I-D276 and II-L39 are also shown.

ascorbate. This generates a highly reactive oxygen species coordinated to the iron, which attacks accessible carbonyl carbons and causes peptide bond hydrolysis. It has been shown that this chemistry results in the transfer of an oxygen atom from H_2O_2 or O_2 to the newly formed C-terminal carboxyl group (Rana & Meares, 1991b).

Since direct contact is required between the Fe−BABE reagent and the carbonyl carbon, the reaction is limited to peptide bonds that are within ≈12 Å of the site of attachment of Fe−BABE. Because of this distance limitation and the required orientation for nucleophilic attack on the peptide carbon, it is expected that the number of cleaved sites will be relatively small, as observed here. The yield of products can be quite high (30%−50% here), providing sufficient material for sequence analysis. This is accomplished by separating the peptide fragments by SDS−PAGE, blotting to PVDF membranes, and sequencing the resulting bands (Matsudaira, 1987).

We have used this approach to address a problem concerning the structure of the cytochrome *bd* ubiquinol oxidase from *Escherichia coli*. Cytochrome *bd* is one of the two respiratory oxidases in *E. coli*, and it catalyzes the two-electron oxidation of ubiquinol in the cytoplasmic membrane and the four-electron reduction of dioxygen to water (Trumpower & Gennis, 1994). Turnover of the enzyme results in generating a transmembrane voltage gradient (Miller & Gennis, 1985) and in the net translocation of one proton per electron across the membrane (Puustinen et al., 1991). This enzyme is the only well characterized respiratory oxidase that is not a member of the heme−copper oxidase superfamily (García-Horsman et al., 1994). The equivalent enzyme from *Azotobacter vinelandii* has been characterized (Kolonay et al., 1994) and appears to be required for aerobic nitrogen fixation (Hill et al., 1990).

Cytochrome *bd* is a heterodimeric enzyme (Miller et al., 1988), consisting of one copy each of subunit I (CydA, 58 kDa) and subunit II (CydB, 43 kDa), and contains three heme prosthetic groups: heme b_{558}, heme b_{595}, and heme *d* (Lorence et al., 1984, 1986). The topology of the two subunits within the *E. coli* membrane has been proposed (Newton et al., 1991) (Figure 1) on the basis of proteolysis experiments (Dueweke & Gennis, 1991), antibody binding (Dueweke & Gennis, 1990), and gene fusions (Newton et al., 1991). Subunits I and II are proposed to contain respectively seven and eight membrane-spanning helices. It has been shown that the binding of monoclonal antibodies to an epitope within the "loop" connecting putative transmembrane helices E and F in subunit I results in abolishing the ability to oxidize quinol (Dueweke & Gennis, 1990). Similarly, proteolysis at sites within this region, known as the Q loop, also results in the specific blockage of quinol oxidase activity (Lorence et al., 1988). On the basis of these data, it has been suggested that portions of the Q loop are likely to be directly involved in forming the quinol-binding site. The immediate electron acceptor from quinol is thought to be heme b_{558} (Hata-Tanaka et al., 1987; Hill et al., 1994; Lorence et al., 1987), and this is located within subunit I (Green et al., 1984). The other two heme prosthetic groups, heme b_{595} and heme *d*, appear to be located within a common binding pocket (Hill et al., 1993), and these two hemes may both participate in the enzymatic oxygen chemistry. Both subunits are required for the assembly of heme b_{595} and heme *d*, suggesting that the binding pocket containing these two hemes may reside at the subunit interface (Newton & Gennis, 1991). This is also suggested by the lability of both of these hemes in several randomly generated mutations in either subunit I or II (Oden & Gennis, 1991). These studies have focused attention on the need for information about the interaction between the two subunits, and for this reason the utility of Fe−BABE was explored.

MATERIALS AND METHODS

Materials. The restriction enzymes *Eco*RI, *Hin*dIII, and *Sal*I were purchased from Bethesda Research Laboratories. T4 DNA ligase and T4 polynucleotide kinase were purchased from New England Biolabs. T4 DNA polymerase, the T7-Gen *in vitro* mutagenesis kit, and the sequenase kit were obtained from U.S. Biochemical Corp. The Wizard minipreps DNA purification systems kit was purchased from Promega Corp. ATP, HEPES, and phenylmethanesulfonyl fluoride were purchased from Sigma. SeaPlaque low melting point agarose was obtained from FMC Bioproducts. Leupeptin was obtained from Boehringer Mannheim. [α-^{35}S]-dATP was purchased from New England Nuclear. Standards for PAGE analysis were purchased from Bio-Rad. The bicinchoninic acid protein assay kit was purchased from Pierce. Thiol groups were determined using DTNB purchased from Aldrich (Ellman, 1959). Synthesis of all oligonucleotides used to sequence or to generate mutations and peptide sequencing were done by the Genetic Engineering Facility at the University of Illinois Biotechnology Center, Urbana, IL. 1-[*p*-(Bromoacetamido)benzyl]EDTA (BABE) was synthesized as described (DeRiemer et al., 1981).

Bacterial Strains. E. coli HB101 [*hsdS20, recA13, ara-14, proA2, lacY1, galK2, rpsL20 (Sm^r), xyl-5, mtl-1, supE44*] (Boyer & Roulland-Dussoix, 1969) was used as host for plasmids described for molecular genetics manipulations. *E. coli* GO105 [*cydAB::kan, cyo, recA*] was the host for expression of a plasmid-encoded cytochrome *bd* since this strain lacks chromosomal expression of both of the repiratory oxidases (Kaysser et al., manuscript in preparation).

Oligonucleotide-Directed Mutagenesis. Oligonucleotide-directed mutagenesis was performed using the T7-Gen *in vitro* mutagenesis system from U.S. Biochemical Corp. Oligonucleotides designed for this method which contained

either an *Msp*I or *Hha*I site within the sequence were methylated at the appropriate dCTP to avoid restriction within the primer. Double-stranded DNA prepared by Wizard minipreps (Promega) was sequenced by using the dideoxy chain termination method (Sanger et al., 1977) and synthetic sequencing primers after alkaline denaturation.

Complementation Analysis of the Mutant Plasmids. The ability of the plasmid-encoded mutant oxidases to complement a strain unable to grow under aerobic conditions was tested using the strain GO105 (Kaysser et al., manuscript in preparation). Mutant plasmids were transformed into anaerobically grown GO105, and transformants were grown anaerobically for selection of ampicillin resistance without selective pressure on the plasmid-carried oxidase operon. Strains exhibiting ampicillin resistance were restreaked and grown anaerobically to colony purify. Aerobic growth of four different isolates for each mutation was then tested on M63 minimal medium (Cohen & Rickenberg, 1956), 0.3% lactate, and 0.3% succinate plates supplemented with 100 μg/mL ampicillin and 50 μg/mL kanamycin. Incubation for 48−72 h at 37 °C was required to define complementation of the mutant plasmid to aerobic growth.

Membrane Preparation and Protein Purification. Twenty liters of each of the mutant oxidases that complemented the anaerobic strain GO105 was grown overnight aerobically at 37 °C in LB medium suplemented with 0.3% lactate, 100 μg/mL ampicillin, and 50 μg/mL kanamycin. All mutant oxidases were purified as described (Miller & Gennis, 1983) with the following modifications. The cells were broken by passing through a microfluidizer (Microfluidics, Newton, MA) three times rather than breaking with a French pressure cell. The solublization buffer contained sulfobetaine-12, or SB-12, equivalent to the Zwittergent detergent used previously. The fractions pooled from the Bio-Rad DNA-grade hydroxyapatite (HAP) column were concentrated and dialyzed for 24 h with two changes of 4−6 L of 10 mM sodium phosphate, 5 mM EDTA, and 0.05% sarcosyl, pH 7.5. Leupeptin (0.5 mg/mL) and a small amount of PMSF were added to the buffers at several steps throughout the purification to minimize proteolysis.

Visible Spectroscopy and Oxidase Activity Assays. Visible spectra were taken on a DW2000 spectrophotometer (SLM-Aminco). Membranes were diluted for spectra in 50 mM potassium phosphate and 1 mM EDTA, pH 7.4, whereas the purified mutants were diluted in 10 mM sodium phosphate, 1 mM EDTA, and 0.05% sarcosyl, pH 7.5. The cytochromes were reduced by addition of a small amount of dithionite. Oxygen uptake measurements were obtained using a Clark-type oxygen electrode apparatus (Yellow Springs Instrument Co., Yellow Springs, OH). Oxygen uptake was initiated by addition of the oxidase preparation to 1.8 mL of 25 mM Tris, 1 mM EDTA, and 2 mM dithioerythritol, pH 7.5, preincubated with ubiquinol-1 at 37 °C. For pure protein samples, 0.05% Triton X-100 was included in the buffer. Ubiquinol-1 (kindly provided by Hoffman-LaRoche) was added to a final concentration of 245 μM. Activities were determined assuming a value of 237 μmol of O_2/L for saturated buffer at 37 °C.

Site-Specific Conjugation of the Mutant Oxidases to Fe−BABE. Alkylation of each of the cyteine mutants of the oxidase with Fe−1-[*p*-(bromoacetamido)benzyl]-EDTA (Fe−BABE) (DeRiemer et al., 1981) was carried out by addition of 10 μL of 20 mM BABE to 200 μL of 80 μM oxidase, followed by incubation for 45 min at 25 °C. The loss of free sulfhydryls was monitored by subjecting aliquots of the reaction mixture to the Ellman's test (Ellman, 1959). After the completion of the alkylation reaction, chelation of iron by the protein-BABE adduct was carried out by addition of 10 μL of freshly prepared 20 mM ferrous sulfate solution to 100 μL of BABE-conjugated protein and allowing it to stand at room temperature for 45 min. Excess free iron was scavenged by addition of 30 μL of 20 mM EDTA (50 mM sodium phosphate buffer, pH 7.4).

Cleavage Reaction. The cleavage reaction was carried out for ~10 s at 25 °C. The reaction was initiated by mixing the modified protein with 50 mM *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonate (HEPES) buffer, pH 7.4, containing 10 mM ascorbate and 5 mM H_2O_2. The reaction was stopped by addition of 50 μL of SDS−PAGE sample application buffer. Denatured samples were analyzed on a 4%−20% gradient SDS−PAGE (Laemmli, 1970).

Cleavage Product Characterization. Once the reaction was stopped, samples were analyzed on a 4%−20% gradient SDS−PAGE (Laemmli, 1970) and blotted onto PVDF membranes (Matsudaira, 1987). N-Terminal sequence analysis of the excised bands was performed on an Applied Biosystems 477A pulsed-liquid sequencer at the Genetics Enginering Laboratory, University of Illinois.

RESULTS

Cytochrome *bd* contains six endogenous cysteines, three each in subunits I and II (Green et al., 1988). However, none of these is reactive to DTNB [5,5′-dithiobis(2-nitrobenzoic acid), Ellman's reagent] (Ellman, 1959), and none of the endogenous cysteines appears to react with Fe−BABE. Hence there was no need to remove endogenous cysteines in cytochrome *bd* before proceeding. Four mutants were prepared, with cysteines replacing S157, D276, and Y509 in subunit I and L39 in subunit II. These residues were selected to examine the proximity relationships between the four hydrophilic regions in which the residues are located (see Figure 1). All four regions are predicted to be on the periplasmic side of the membrane. It has been proposed (Oden & Gennis, 1991) that the N-terminus of subunit II might be involved in the subunit interface, based on the behavior of a mutant, II-G69D, in which heme b_{595} and heme *d* appear to be destabilized. II-L39C was made to examine this possibility.

All four mutant oxidases were purified to homogeneity and shown to have wild-type enzymatic and spectroscopic characteristics. Using Ellman's reagent, I-D276C, I-Y509C, and II-L39C each have between 0.7 to 1.2 reactive cysteines per oxidase, but the cysteine introduced in I-S157C appears to be unreactive and is concluded to be buried. Site-specific conjugation of the purified I-D276C, I-Y509C, and II-L39C with BABE was monitored by the loss of the reactive thiol group (Rana & Meares, 1991b). Formation of the adducts in each case has no effect on either the quinol oxidase activity of the enzymes or their spectroscopic properties (data not shown). The specific activities of the II-L39C and I-D276C mutants are 92% and 80%, respectively, of the wild type (600 e^-/s).

The hydrolysis of cytochrome *bd* adducts with Fe−BABE was initiated by the addition of ascorbate and H_2O_2. After about 10 s, the reaction was stopped by the addition of the

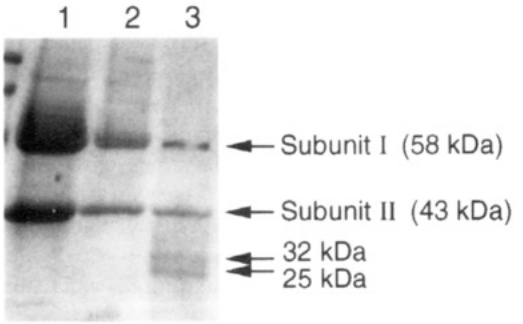

FIGURE 2: Separation of the hydrolysis products of Fe−BABE tethered to I-D276C by SDS−PAGE: I-D276C protein (lane 1); control reaction of I-D276C (without Fe−BABE) exposed for 10 s to 20 mM ascorbate/5 mM H_2O_2 (lane 2); cleavage of Fe−BABE-conjugated I-D276C exposed for 10 s to 20 mM ascorbate/5 mM H_2O_2 (lane 3).

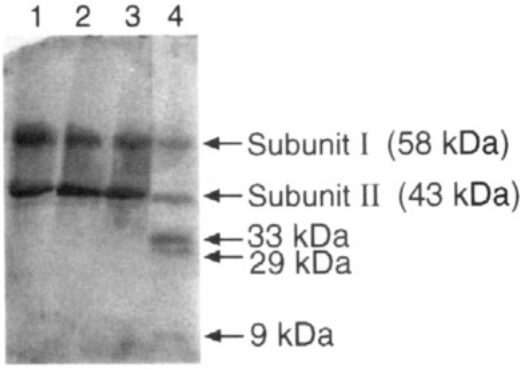

FIGURE 3: Separation of the hydrolysis products of the Fe−BABE adduct of II-L39C by SDS−PAGE: II-L39C protein without Fe−BABE (lane 1); II-L39C without Fe−BABE, exposed for 10 s to 20 mM ascorbate/5 mM H_2O_2 (lane 2); Fe−BABE conjugated to II-L39C in the absence of ascorbate/H_2O_2 (lane 3); cleavage of Fe−BABE conjugated II-L39C upon 10 s exposure to 20 mM ascorbate/5 mM H_2O_2 (lane 4).

SDS−PAGE loading buffer. The peptides were analyzed using a 4%−20% gradient SDS−PAGE system (Laemmli, 1970), followed by electroblotting onto PVDF membranes (Matsudaira, 1987). In the cases of I-D276C and II-L39C the reactions resulted in polypeptide cleavage, but for the I-Y509C mutant, no new bands were detected. For the I-D276C adduct, scission occurs at a single site in subunit I, as shown by the presence of two bands with apparent molecular masses of 32 and 25 kDa (Figure 2). The cleavage from the II-L39C mutant appears to occur at two distinct sites, one from each subunit, resulting in fragments migrating with apparent molecular masses of 33, 29, and 9 kDa (Figure 3). No subunit fragmentation was observed in the absence of ascorbate (needed to reduce the iron), H_2O_2, or the Fe−BABE adduct (Figures 2 and 3). Cleavage is strictly due to the reaction of ascorbate and H_2O_2 with the Fe−BABE−protein conjugate.

Since both of the N-termini of subunits I and II are blocked, only peptides with newly generated N-termini resulting from hydrolysis are apparent. For the I-D276C mutant, the N-terminal sequence of the 25 kDa peptide (Figure 2), Val-Asp-Thr-Pro-Val-Ile-Gly-Leu, is uniquely located at residues 300−307 of subunit I. The second fragment (32 kDa) resulting from this reaction is not expected to have a free N-terminus, and this was confirmed. The cleavage site (I-S299/V300) is shown in the schematic in Figure 1 and is 23 residues from I-D276C, located within the same hydrophilic region.

Analysis of the 29 kDa peptide from the II-L39C adduct (Figure 3) yielded a unique N-terminal sequence, Ile-Glu-Ala-Glu, which corresponds to residues 256−259 of subunit I. Thus, hydrolysis occurs between residues A255 and I256, generating a 265 amino acid fragment extending to the C-terminus of subunit I. The N-terminal half of subunit I expected from this reaction has virtually the same molecular weight as does the C-terminal fragment, so they will migrate identically in this SDS−PAGE system. Hence, this peptide is not resolved on the gel. Since the N-terminus of subunit I is blocked, the presence of this fragment is not apparent. Figure 1 shows that this cleavage site is within the Q loop of subunit I, connecting helices E and F.

The N-terminal sequence of the 33 kDa peptide (Figure 3), Ala-Gly-Gly-Ala-Leu, is uniquely located at residues 67−71 of subunit II of the complex. Therefore, hydrolysis occurs between residues T66 and A67, generating a 314 amino acid peptide extending to the C-terminus of subunit II. The 9 kDa peptide does not yield any sequence, as expected since the N-terminus of subunit II is blocked. This cleavage site (II-T66/A67) is shown in Figure 1 to be within the same interhelical region and 37 residues from the location of the Fe−BABE (II-L39).

DISCUSSION

Two of the four cysteines introduced into cytochrome *bd* reacted with Fe−BABE and produced hydrolytic fragments upon activation of the iron chelate. In each case, one cleavage site was found within the same interhelical loop containing the cysteine to which the Fe−BABE was attached. Evidently this reflects the local folding of these regions. Of greater interest is the hydrolysis by Fe−BABE, tethered to L39 in the first periplasmic loop of subunit II, of a peptide bond located in the Q loop, which is the third periplasmic loop of subunit I. This is consistent with the proposed topology and also indicates that these two regions of the enzyme are part of—or adjacent to—the subunit interface. It is evident that this procedure can be used to derive numerous other distance constraints to contribute to a structural model of cytochrome *bd*.

The peptide bonds observed to be hydrolyzed here are Ala−Ile, Thr−Ala, and Ser−Val. In earlier work Ser−Ser, Ala−Pro, and Leu−Asp bonds were hydrolyzed (Rana & Meares, 1990, 1991a). These six different dipeptides contain eight different amino acids, possessing hydrophobic, hydrophilic, charged, branched, and cyclic side chains.

Fe−BABE is a powerful probe of protein structure, usable with relative ease in any system where cysteine-linked reagents are applicable. As shown here it is particularly well suited to the study of proximity relationships in multisubunit proteins. The cleavage reaction is fast (seconds), highly selective, and proceeds in high yield under physiological conditions. It forms hydrolytic products that are readily characterized by standard N-terminal sequencing of the resolved peptides. The results provide direct information on the proximity of the tethered probe to particular *peptide bonds* regardless of amino acid residue type, easily exceeding the scope and resolution of chemical cross-linking reagents.

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