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A two-domain structure for the two subunits of NAD(P)H:quinone acceptor oxidoreductase

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Abstract

NAD(P)H:quinone acceptor oxidoreductase (EC 1.6.99.2) (DT-diaphorase) is a FAD-containing reductase that catalyzes a unique 2-electron reduction of quinones. It consists of 2 identical subunits. In this study, it was found that the carboxyl-terminal portion of the 2 subunits can be cleaved by various proteases, whereas the amino-terminal portion cannot. It was also found that proteolytic digestion of the enzyme can be blocked by the prosthetic group FAD, substrates NAD(P)H and menadione, and inhibitors dicoumarol and phenindione. Interestingly, chrysin and Cibacron blue, 2 additional inhibitors, cannot protect the enzyme from proteolytic digestion. The results obtained from this study indicate that the subunit of the quinone reductase has a 2-domain structure, i.e., an amino-terminal compact domain and a carboxyl-terminal flexible domain. A structural model of the quinone reductase is generated based on results obtained from amino-terminal and carboxyl-terminal protein sequence analyses and electrospray mass spectral analyses of hydrolytic products of the enzyme generated by trypsin, chymotrypsin, and *Staphylococcus aureus* protease. Furthermore, based on the data, it is suggested that the binding of substrates involves an interaction between 2 structural domains.

Keywords: active site; FAD prosthetic group; proteolysis; quinone reductase

NAD(P)H:quinone acceptor oxidoreductase (EC 1.6.99.2), also referred to as DT-diaphorase, is a flavoprotein that catalyzes the 2-electron reduction of quinones and quinonoid compounds to hydroquinones, using either NADH or NADPH as the electron donor (Iyanagi & Yamazaki, 1970; Iyanagi, 1987). It consists of 2 identical subunits, each 273 amino acids long (Robertson et al., 1986; Bayney et al., 1987; Haniu et al., 1988; Jaiswal et al., 1988) and with a molecular weight of 30 kDa (a detailed discussion of the molecular weight of the enzyme will be given below). In addition, each subunit contains 1 FAD prosthetic group noncovalently attached to the protein (Hosoda et al., 1974).

The quinone reductase plays an important role in protecting tissues against the mutagenic, carcinogenic, and cytotoxic effects of quinones, which occur widely in nature (Benson et al., 1980; Ernster, 1987). In addition, evidence has accumulated that the quinone reductase is involved in the reductive activation process of the cytotoxic antitumor quinones, such as mitomycins, anthracyclines, and aziridinylbenzoquinones, in cells (Siegel et al., 1990a, 1990b; Boland et al., 1991; Walton et al., 1991). Finally, this quinone reductase reduces vitamin K: it can function physiologically as one of several vitamin K reductases in the vitamin K cycling involved in the hepatic posttranslational modification of vitamin K hydroquinone-dependent blood coagulation factors (Wallin et al., 1978). This enzyme is also a target for anticoagulants, such as dicoumarol and warfarin (Hollander & Ernster, 1975; Lind et al., 1979). Although the physiological roles of this enzyme have been gradually unraveled through extensive research, studies on understanding its structure-function relationship and the mechanism of the enzyme in performing the functions described above remain incomplete. A 3-dimensional structure is essential for understanding the mechanism of the enzyme. The X-ray structure of the enzyme is presently being determined. Dr. P. Talalay and his colleagues at Johns Hopkins University are investigating the structure of Cibacron blue-incorporated rat quinone reductase (Ysern & Prochaska, 1989), and Dr. R. Knox and his colleagues at the Institute of Cancer Research, Surrey, UK, are studying the structure of the rat enzyme without substrate (Skelly et al., 1989).

In this study, we obtained results suggesting that each subunit of the quinone reductase has a 2-domain structure and results suggesting that the binding of substrates and inhibitors to the enzyme involves an interaction between 2 structural domains. These findings have important implications concerning the mechanism of the quinone reductase.

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Results and discussion

Characterization of hydrolytic products of NAD(P)H:quinone acceptor oxidoreductase by trypsin, chymotrypsin, or Staphylococcus aureus protease

The quinone reductase is a homodimer. Since the amino-terminus of the *Escherichia coli*-expressed enzyme is not blocked by an acetyl group (Chen et al., 1992) as identified for the rat liver enzyme preparation (Haniu et al., 1988), the molecular weight of each subunit is calculated to be 30,817 based on its amino acid sequence; the molecular weight determined by our electrospray mass spectral analysis was 30,819. As demonstrated by SDS-PAGE, incubation of the quinone reductase (in a non-denatured condition) with trypsin (5%, w/w) results in the formation of 1 major product, a 27-kDa fragment (Fig. 1A). No other major fragmentation was found, even after an overnight incubation with trypsin (discussed below). This observation indicates that the major portion of the enzyme has a compact structure that is resistant to trypsin digestion. More interestingly, hydrolytic products of similar size were obtained when the enzyme was digested with 3 different proteases: trypsin, chymotrypsin, and *S. aureus* protease (Fig. 1).

In order to identify the sites of hydrolysis by the 3 proteases and to determine whether these proteases digest the enzyme in a specific fashion, the following experiments were performed. A trypsin-digested enzyme preparation was initially subjected to an amino-terminal amino acid sequence analysis. The analysis was carried out for 17 cycles and the major sequence was the N-terminal sequence of the quinone reductase, i.e., AVR RALIVLAHAERTSF (the amino acid sequence of the rat quinone reductase is shown in Fig. 2). Four additional sequences were recognized in the analysis: ^{241}EVQEEQK^{247}, ^{251}FGLSV GHHLGK^{261}, ^{262}SIPADNQIK^{270}, and ^{271}ARK^{273}. This result clearly indicates that the trypsin digestion occurred only in the carboxyl-terminal region of the quinone reductase. In order to further characterize the hydrolytic products, HPLC-linked electrospray mass spectral analysis and direct C-terminal amino acid sequence analysis of the hydrolyzed samples were performed (results are summarized in Fig. 2). For the trypsin-digested sample, the major product, an ion with a mass of 26,989, was identified through reverse-phase HPLC-linked electrospray mass spectral analysis. The calculated mass of an enzyme fragment containing amino acid residues 1–239 is 26,991. This fragment was purified through reverse-phase HPLC and subjected to C-terminal sequence analysis. The C-terminal sequence analysis revealed that there is only 1 sequence, and the amino acids in the first 3 cycles were determined to be K, L, and L. The analysis was performed with 300 pmol of the peptide (assuming a 30% recovery of the peptide from HPLC separation). The yields were 3 pmol for K, 2 pmol for L, and 1.5 pmol for L in 3 cycles, respectively. The low yields for the C-terminal sequence analysis may be due, at least in part, to an inefficient binding of the enzyme fragment to the Zitex membrane during the sequence analysis. Based on experience so far, the binding efficiency of sequencing samples varies significantly from one to another. Sample binding conditions are evaluated to improve the binding efficiency during C-terminal sequence analysis. In this C-terminal sequence analysis, only 1 sequence was detected, and the results did agree with those obtained from electrospray mass spectral analysis. Thus, the results indicate that trypsin cleaved the quinone reductase after Lys-239.

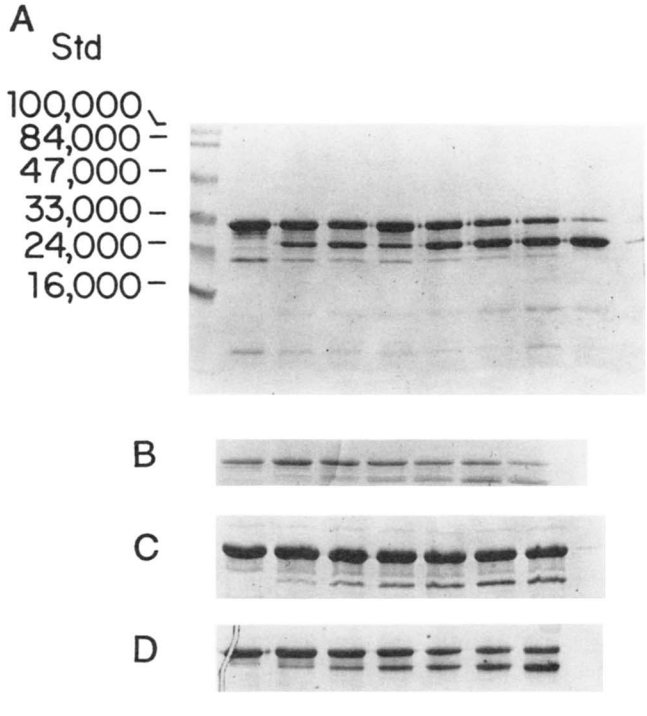

Fig. 1. SDS-polyacrylamide gel electrophoresis of trypsin-, chymotrypsin-, and *S. aureus* protease-digested quinone reductase preparations. **A:** The quinone reductase (118 μg in 200 μL of 50 mM phosphate buffer, pH 7.5) was incubated at 37 °C in the presence of 2.36 μg of trypsin. After 0, 1, 1.5, 2, 3, 4, 6, and 16 h incubation, a 10-μL aliquot was withdrawn and analyzed by SDS-polyacrylamide gel electrophoresis. The 30- and 24-kDa bands are the quinone reductase and trypsin, respectively. **B–D:** The analyses were performed with samples withdrawn after 0, 0.17, 0.5, 1, 1.5, 2, and 2.5 h incubation of the quinone reductase (118 μg in 200 μL) with 2.36 μg of trypsin, chymotrypsin, and *S. aureus* protease, respectively. To save space, in B–D, only the portions of the gels showing the enzyme and its 27-kDa fragments are shown.

For the *S. aureus* protease-digested sample, an ion with a mass of 25,863 was identified by reverse-phase HPLC-linked electrospray mass spectral analysis. An enzyme fragment containing amino acid residues 1–229 would have a mass of 25,859. This fragment had a C-terminal sequence of DFL, indicating that this protease cleaved the enzyme after Asp-229. The C-terminal sequence analysis was performed with 400 pmol of the peptide. Asp was detected in cycle 1, but it was not quantitated because of the broadness of the peak. The yields were 22 pmol for F in cycle 2 and 16 pmol for L in cycle 3.

For the chymotrypsin-digested sample, the major fragment was determined to have a mass of 25,746 by electrospray mass spectral analysis. The calculated mass for a protein fragment containing amino acid residues 1–228 is 25,744. The HPLC fraction that was thought to contain the fragment with the mass of 25,746 was subjected to C-terminal sequencing. The sequence analysis was carried for 3 cycles, and 2 amino acids were found in the first and second cycles. F and Y were the amino acids in the first cycle, L and M in the second cycle, and S in the third cycle. The analysis was performed with 300 pmol of the peptide. The yields were 15 pmol for F and 27 pmol for Y in cycle 1, and 14 pmol for L and 8 pmol for M in cycle 2. Serine was seen in cycle 3, but it was not quantitated. Because of these sequence data, we decided to again analyze this HPLC fraction by elec-

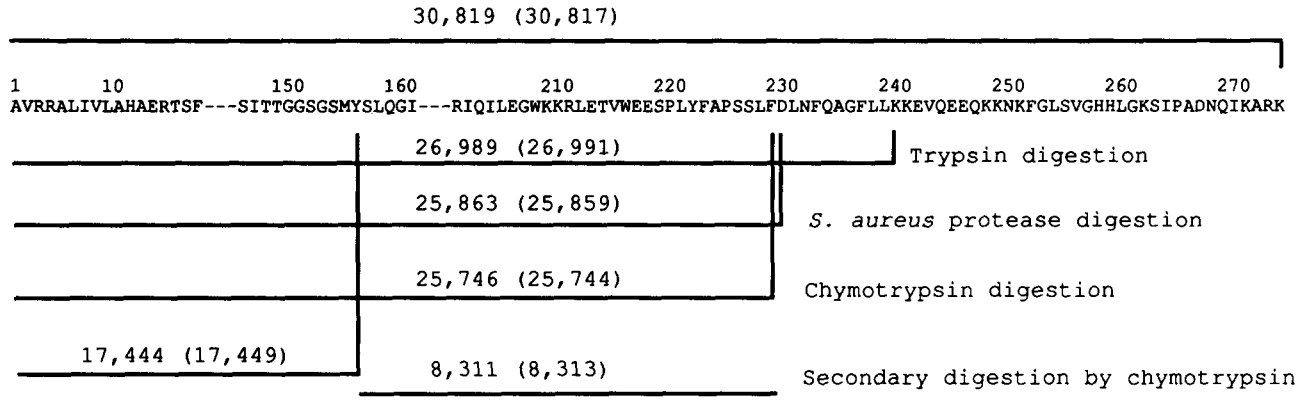

Fig. 2. A summary of the results obtained from electrospray mass spectral and C-terminal amino acid sequencing analyses of the proteolytic digests of the quinone reductase. The molecular weights of the major proteolytic fragments, as determined by electrospray mass spectrometry, are shown. The calculated molecular weights for these fragments are also shown in parentheses.

trospray mass spectrometry. It was found that the fraction actually contained 2 ions with masses of 25,746 and 17,444. A peptide containing amino acid residues 1–155 would have a mass of 17,449. The amino acid residues of the quinone reductase from positions 153 to 155 are S, M, and Y, and amino acid residues from positions 226 to 228 are S, L, and F. This explains why we obtained 2 sequences during the C-terminal sequence analysis. After extensive incubation with chymotrypsin, hydrolytic products with masses of 17,444 and 8,311 (i.e., the peptide containing amino acid residues 156–228) were clearly detected. They are secondary hydrolytic products of the major fragment containing amino acid residues 1–228. The peptide with the mass of 8,311 was purified and subjected to N-terminal as well as C-terminal sequence analyses, confirming that this is the peptide containing amino acid residues 156–228. From these we conclude that chymotrypsin cleaves the enzyme after Phe-228. After extensive incubation with chymotrypsin, the major fragment is further hydrolyzed into 2 peptides at position 155.

Thus, these results indicate that the 3 proteases all cleave the quinone reductase at the carboxyl-terminal region and in a rather specific manner. Based on these findings, we propose that each subunit of the enzyme is composed of 2 domains, a rigid N-terminal domain and a flexible C-terminal domain. Additional minor products were obtained after extensive incubation with all 3 proteases and could be seen upon SDS gel or HPLC separation. No conclusion will be made until all of them are evaluated.

Characterization of the trypsin-digested quinone reductase preparation

The quinone reductase migrated as 1 peak, with full activity, when passed through a size-exclusion HPLC column under nondenaturing conditions, and the molecular weight of the enzyme was estimated to be 52 kDa by this analysis. Although the estimated molecular weight was somewhat lower than that for a dimer form of the quinone reductase (i.e., 60 kDa), considering the nature of the analysis, we feel that this result still supports a conclusion that the enzyme is indeed a dimer. Using the same method, the trypsin-digested enzyme migrated as 1 peak with a molecular weight estimated to be 48 kDa. As indicated by SDS-PAGE, this fraction contained the 27-kDa fragment and a trace amount of the intact enzyme. The results indicate that the enzyme still remains in dimer form even when the C-terminal domain is cleaved off. The UV/visible (UV/vis) spectrum of the fraction containing the trypsin-digested fragment revealed that it still contained the FAD prosthetic group (Fig. 3), although in a significantly reduced amount. The released FAD was separated by size-exclusion HPLC and detected based on its UV/vis spectrum. The purified hydrolytic fragment preparation had 9% of the control activity, and the activity could not be recovered by the addition of FAD to the preparation or by including FAD during the assay. These results suggest that removal of the C-terminal domain causes a loss of the FAD prosthetic group and a loss of the enzyme activity. The residual FAD and residual activity associated with the digested preparation are due, at least in part, to a small amount of undigested enzyme in the preparation. Because the 27-kDa fragment preparation still has a substantial amount of FAD (certainly more than 9% of the control; see Fig. 3), it is thought that FAD would bind to the

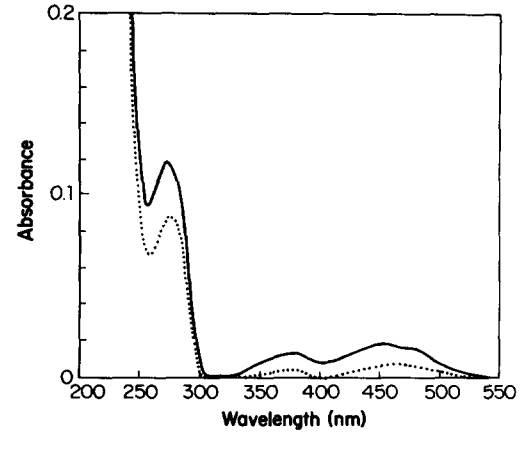

Fig. 3. UV/vis spectra of intact and trypsin-digested quinone reductase preparations. Size-exclusion HPLC was carried out with 55 μg of the enzyme. The peak fractions containing the enzyme (——) and its hydrolytic product (· · · · ·) were diluted to 1 mL and their UV/vis spectra were recorded.

27-kDa fragment but with a lower affinity than to the intact enzyme. We are making attempts to prepare the 27-kDa fragment in a pure form for further characterization of its catalytic properties.

Digestion of the apoenzyme with trypsin

Through collaboration with Dr. V. Massey at the University of Michigan, a procedure was developed to remove the FAD prosthetic group from the enzyme in a nondenaturing manner (the procedure for the generation of apoenzyme will be published elsewhere; briefly, the apoprotein was obtained by dialyzing the holoenzyme at 4 °C against 200 mM potassium phosphate, pH 6.0, containing 2 M KBr). The enzyme activity could be recovered upon addition of FAD to this apoenzyme preparation. We observed several things when the apoenzyme was digested with trypsin using conditions identical to those for the whole enzyme preparations. Firstly, 3 major hydrolytic products were found when the apoenzyme was treated with trypsin (see Fig. 4A). The masses of these products were determined to be 29,496, 28,118, and 27,116 by electrospray mass spectral analysis, indicating that the hydrolytic products were a fragment containing amino acid residues 1–261 (calculated MW 29,494), a fragment containing amino acid residues 1–248 (calculated MW 28,118), and a fragment containing amino acid residues 1–240 (calculated MW 27,119), respectively. This analysis confirms again that the quinone reductase has a rigid N-terminal domain (i.e., the enzyme was not digested further, into peptides smaller than 27 kDa, even without the FAD prosthetic group). The results suggest that FAD is not embedded in the N-terminal domain. Secondly, since we did not detect the presence of an enzyme fragment containing amino acid residues 1–270, the peptide ^{262}SIPADNQIK^{270} is probably rapidly cleaved off by trypsin. Thirdly, as shown in Figure 4A, trypsin seems to hydrolyze the enzyme from the C-terminus in a sequential manner. Fourthly, the tryptic hydrolysis of the apoenzyme proceeded significantly faster than that of the whole enzyme preparation (cf. Fig. 1). There was no intact enzyme left after 1 h incubation.

As a control experiment, we also carried out trypsin hydrolysis of the FAD-reconstituted enzyme preparation. As indicated in Figure 4B, while we still obtained the same 3 products, the hydrolysis was slowed down significantly for the FAD-reconstituted preparation. These results indicate that FAD protects the enzyme from digestion by trypsin. Moreover, these results suggest that the C-terminal domain plays a role in the binding of the FAD prosthetic group to the enzyme.

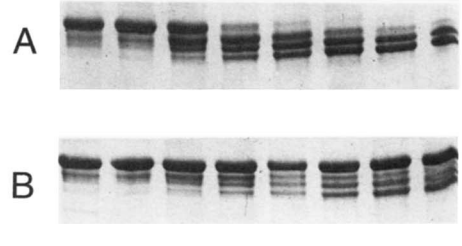

Fig. 4. SDS-polyacrylamide gel electrophoresis of the trypsin-treated apoenzyme (**A**) and FAD-reconstituted enzyme (**B**) preparations. The first lane contains the sample without trypsin treatment. Lanes 2–8 are samples withdrawn after 0, 0.17, 0.5, 1, 1.5, 2, and 2.5 h incubation of the quinone reductase (118 μg in 200 μL) with 2.36 μg of trypsin.

Protection of trypsin digestion of the quinone reductase by substrates and inhibitors of the enzyme

The involvement of the C-terminal domain of the quinone reductase in catalysis was further confirmed by the following experiments. As shown in Figure 5A and B, the tryptic digestion of the enzyme could be prevented by including NAD(P)H or menadione during the incubation. The protection was in a concentration-dependent manner. NAD(P)H and menadione are substrates of the quinone reductase. Dicoumarol, a very potent competitive inhibitor of this enzyme with respect to NAD(P)H (Ernster et al., 1962), also protected the quinone reductase from digestion by trypsin (Fig. 5C). In addition, phenindione, another competitive inhibitor of the quinone reductase with respect to NAD(P)H but with a binding affinity of 1/50 of dicoumarol (Hollander & Ernster, 1975), also protected the trypsin digestion of the enzyme by using a concentration of 50 times of that of dicoumarol (Fig. 5D). These results suggest that a conformational change may occur during the binding of substrates and the 2 inhibitors to the quinone reductase, i.e., upon binding of these ligands, the C-terminal domain has a conformation such

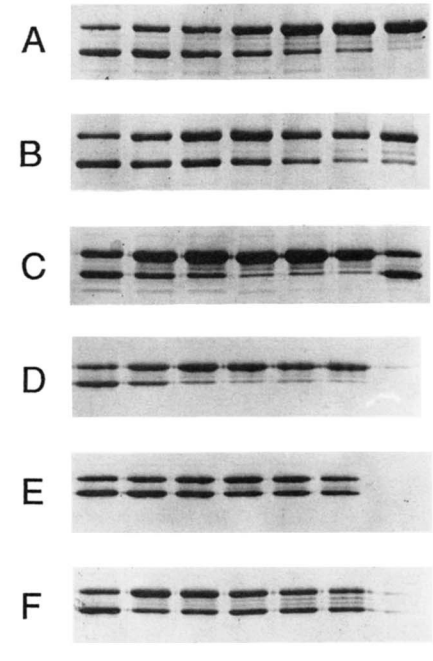

Fig. 5. SDS-polyacrylamide gel electrophoresis of the quinone reductase samples (each sample contained 7.4 μg enzyme in 25 μL of 50 mM phosphate buffer, pH 7.5) that were treated with trypsin (at 5% [w/w] for 2.5 h at 37 °C) in the presence of its substrates or inhibitors. **A:** Samples were prepared by digestion with trypsin in the presence of 0, 0.38, 0.76, 1.53, 3.05, 5.42, or 7 mM NADPH. **B:** Samples were prepared by digestion with trypsin in the presence of 0, 0.075, 0.15, 0.31, 0.61, 1.08, or 1.4 mM menadione. **C:** Samples were prepared by digestion with trypsin in the presence of 0, 7.4, 14.2, 26.5, 42.2, or 55.2 μM dicoumarol. Because dicoumarol was dissolved in 5 mM NaOH, the last sample in this series contained 1.4 mM NaOH. This is a control sample, and the result shows that the digestion is not affected by this amount of NaOH (the highest level in this set of experiments). **D:** Samples were prepared by digestion with trypsin in the presence of 0, 184, 355, 662, 1,054, and 1,381 μM phenindione. **E:** Samples were prepared by digestion with trypsin in the presence of 0, 37, 71, 132, 211, and 276 μM chrysin. **F:** Samples were prepared by digestion with trypsin in the presence of 0, 37, 71, 132, 211, and 276 μM Cibacron blue.

that it becomes resistant to the protease treatment. Furthermore, these results imply an interaction between the N-terminal and C-terminal domains of the quinone reductase when substrates bind to the enzyme (see below). The protection of trypsin digestion of the quinone reductase by its substrates and inhibitors was not due to inhibition of trypsin by these compounds. As a control experiment, at the highest concentrations used in this study, these compounds, except NADPH, did not affect the digestion of cytochrome *c* by trypsin. At the highest concentration, NADPH had a weak inhibitory effect on the digestion of cytochrome *c* by trypsin, but it fully protected the quinone reductase from trypsin digestion at this concentration (see Fig. 5).

Cibacron blue and chrysin (5,7-dihydroxylflavone) are also potent competitive inhibitors of this quinone reductase with respect to NAD(P)H (Liu et al., 1990; Prestera et al., 1992). They have binding affinities that are 1/5 and 1/10 of that for dicoumarol, respectively. Using concentrations that are 5 times those of dicoumarol, no clear protection of the enzyme from digestion by trypsin was observed (Fig. 5E,F). These results suggest that Cibacron blue and chrysin bind to the enzyme differently from dicoumarol. In a previous inhibition kinetic study, we showed that flavone derivatives such as chrysin bind to a site different from that for dicoumarol or phenindione (Chen et al., 1993).

A proposed model for the active site of NAD(P)H:quinone acceptor oxidoreductase

Based on the results presented in this paper and those generated previously in this and other laboratories, a model of the active site of the quinone reductase is proposed. As described above, the quinone reductase is a homodimer and each subunit has a 2-domain structure—an N-terminal compact domain and a C-terminal flexible domain. A secondary structure prediction (Chou & Fasman, 1978) suggests that the region containing amino acid residues 215–230 is a turn, and we have proposed that this is the hinge region between the 2 domains. As indicated here, 3 proteases with different specificities all cleave the quinone reductase in or near this turn region. The rest of the C-terminus may have a more ordered structure. Secondary structure analysis suggests that the regions containing amino acid residues 231–240 and amino acid residues 255–265 have a β-sheet structure, and the region containing amino acid residues 240–255 has an α-helix structure.

The results indicate that the C-terminal domain of the quinone reductase is important for its catalysis because the purified 27-kDa fragment (by size-exclusion HPLC) has a significantly lower activity than the intact enzyme preparation. Jaiswal et al. (1990) reported the isolation of a cDNA clone NQO_2 from a human liver cDNA library. NQO_2 encodes a protein with a sequence homologous to the quinone reductase but lacking the C-terminal portion of the quinone reductase. The COS-1 monkey tumor kidney cell-expressed NQO_2 protein was found to have a very low quinone reductase activity (Jaiswal et al., 1990). It is possible that the low catalytic activity for the NQO_2 protein is due, at least in part, to a lack of the C-terminal portion of the quinone reductase.

The binding of the FAD prosthetic group was found in this study to be destabilized upon the removal of the C-terminal domain; through experiments with apoenzyme and reconstituted enzyme preparations, FAD was found to protect the enzyme from tryptic digestion. Furthermore, substrates, e.g., NADPH and menadione, were found to protect the enzyme from proteolytic digestion. These results indicate that the C-terminal domain plays an important role for the binding of FAD and substrates. However, it is also possible that binding of substrate/prosthetic group/inhibitors in the N-terminal domain results in a folding of the C-terminal domain to make it less available to protease. Site-directed mutagenesis experiments at the C-terminal domain will be important for further evaluation of the importance of this region of the enzyme.

Considering that the C-terminal domain may be important for the binding of FAD and substrates, and that several functionally important regions have been identified on the N-terminal domain (see further discussion below), we propose that the active site of the quinone reductase is situated between the 2 structural domains. Furthermore, we believe that the enzyme active site has different conformations when NAD(P)H or quinone binds to the active site of the enzyme. In a previous affinity-labeling study using 5′-(*p*-(fluorosulfonyl)benzoyl)-adenosine (5′-FSBA), reverse-phase HPLC of tryptic digests of [^{14}C]5′-FSBA-labeled quinone reductase revealed 1 radioactive peak containing 2 peptides, ^{146}ITTGGSGSMY^{155} and ^{262}SIPAD NQIK^{270} (Liu et al., 1989). By comparing the sequences to those of the nucleotide binding sites of several kinases and dehydrogenases (as well as a secondary structure prediction [Forrest et al., 1990]), it was suggested that the peptide ^{146}ITTGGS GSMY^{155} was the one modified by 5′-FSBA and would be in the region where the pyrophosphate group of NAD(P)H binds (Liu et al., 1989). Through amino acid sequence alignment analysis, this region was also suggested to be a part of the nucleotide binding site by Prochaska (1988). This was further confirmed recently by characterizing quinone reductase mutants with changes in this region (Ma et al., 1992). To further support these results, we found that, as discussed above, there was a secondary hydrolysis at Tyr-155 by chymotrypsin after extensive incubation. This tyrosine residue is the one at the proposed region where the pyrophosphate group of NAD(P)H binds. It is not unreasonable to think that this region is partially exposed and can be attacked by chymotrypsin, especially after the C-terminal domain is removed. The question is raised as to why trypsin or *S. aureus* protease was not able to hydrolyze the enzyme at this region after extensive incubation. The answer lies in the fact that there is not a suitable Lys (or Arg) or Glu (or Asp) residue in this region that can be attacked by these proteases (see Fig. 2).

It is thought that the FAD prosthetic group binds to the enzyme between the 2 structural domains. Based on results obtained from a recent NMR analysis of the enzyme, FAD is thought to bind to the enzyme with the pyrophosphate group exposed to an unrestricted environment (unpubl. results).

When the quinone substrate binds to the quinone reductase, the active site is thought to be in a conformation different from that when NAD(P)H binds, resulting in a high affinity for quinone and hydroquinone. The release of the hydroquinone from the active site has been suggested to be the rate-limiting step of the reaction catalyzed by the quinone reductase (Hosoda et al., 1974; Chen & Liu, 1992). Less is known concerning the quinone binding site. Tyr-128 has been suggested to be a part of the quinone binding site based on our affinity labeling experiments (Haniu et al., 1988) and recent site-directed mutagenesis experiments (Ma et al., 1992). This residue is situated in an aromatic amino acid-enriched region that is thought to be important for the binding of quinone.

In summary, this study reveals that each subunit of the quinone reductase has a 2-domain structure. This information is important for our future structure–function studies of the enzyme. X-ray structural analysis of the quinone reductase is essential to unravel the structure of the C-terminal domain and to verify our 2-domain model. While we are waiting for the X-ray structural information, we are carrying out various structure–function studies to further examine the role of the C-terminal region of the quinone reductase during enzyme catalysis.

Materials and methods

Materials

The study was carried out using an *E. coli*-expressed rat liver NAD(P)H:quinone acceptor oxidoreductase that was prepared as described by Chen et al. (1992). The specific activity of the enzyme preparation is 7,670 μmol/min per mg.

Trypsin and chymotrypsin were purchased from Sigma. These proteases were purified through reverse-phase HPLC before they were used. *S. aureus* protease was obtained from Boehringer Mannheim. NAD(P)H, menadione, dicoumarol, phenindione (2-phenyl-1,3-indandione), chrysin (5,7-dihydroxylflavone), and Cibacron blue were from Sigma.

Methods

In this study, the enzyme activity was determined spectrophotometrically by measuring the reduction of 3-(4,5-dimethylthiazo-2-yl) 2,5-diphenyltetrazolium bromide (MTT) at 610 nm as described in Chen et al. (1993). The N-terminal amino acid sequence analyses were carried out by a procedure similar to that published by Ronk et al. (1991). The C-terminal sequence analyses were performed using an automated chemical method developed at the City of Hope (Bailey et al., 1992). The present study represents the first time that useful structural information has been generated by C-terminal sequence analysis of a research sample. The HPLC-linked electrospray mass spectral analyses were performed by a method described in 2 previous publications from our division (Davis & Lee, 1992; Swiderek et al., 1992). SDS-PAGE was performed by the method of Laemmli (1970).

HPLC conditions

The proteolytic digests were subjected to reverse-phase HPLC using a Pierce RP300 column (2 × 30 mm). The peptides were eluted with a linear 30-min gradient from 100% solvent I (0.1% trifluoroacetic acid [TFA]) to 100% solvent II (TFA/water/acetonitrile, 0.1:9.9:90). Peptides were detected by absorbance at 214 nm and were collected manually. Size-exclusion HPLC was performed using a DuPont GF250 column. The enzyme was eluted with 200 mM phosphate buffer, pH 7.4. The protein standards used in this analysis were thyroglobulin (MW 670,000), bovine gamma globulin (MW 158,000), chicken ovalbumin (MW 44,000), equine myoglobin (MW 17,000), and vitamin B-12 (MW 1,350).

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