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Mouse liver NAD(P)H:quinone acceptor oxidoreductase: Protein sequence analysis by tandem mass spectrometry, cDNA cloning, expression in *Escherichia coli*, and enzyme activity analysis

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Abstract

The amino acid sequence of mouse liver NAD(P)H:quinone acceptor oxidoreductase (EC 1.6.99.2) has been determined by tandem mass spectrometry and deduced from the nucleotide sequence of the cDNA encoding for the enzyme. The electrospray mass spectral analyses revealed, as previously reported (Prochaska HJ, Talalay P, 1986, *J Biol Chem 261*:1372-1378), that the 2 forms—the hydrophilic and hydrophobic forms—of the mouse liver quinone reductase have the same molecular weight. No amino acid sequence differences were found by tandem mass spectral analyses of tryptic peptides of the 2 forms. Moreover, the amino-termini of the mouse enzymes are acetylated as determined by tandem mass spectrometry. Further, only 1 cDNA species encoding for the quinone reductase was found. These results suggest that the 2 forms of the mouse quinone reductase have the same primary sequences, and that any difference between the 2 forms may be attributed to a labile posttranslational modification.

Analysis of the mouse quinone reductase cDNA revealed that the enzyme is 273 amino acids long and has a sequence homologous to those of rat and human quinone reductases. In this study, the mouse quinone reductase cDNA was also ligated into a prokaryotic expression plasmid pKK233.2, and the constructed plasmid was used to transform *Escherichia coli* strain JM109. The *E. coli*-expressed mouse quinone reductase was purified and characterized. Although mouse quinone reductase has an amino acid sequence similar to those of the rat and human enzymes, the mouse enzyme has a higher NAD(P)H-menadione reductase activity and is less sensitive to flavones and dicoumarol, 2 known inhibitors of the enzyme. The results would indicate that the regions in mouse quinone reductase that contain amino acids different from the rat and human enzymes are critical for the binding of menadione, flavones, and dicoumarol.

Keywords: amino acid sequence; cDNA; mouse liver quinone reductase; tandem mass spectrometry

NAD(P)H:quinone acceptor oxidoreductase (EC 1.6.99.2), also referred to as DT-diaphorase, is a flavoprotein that catalyzes obligatory 2-electron reduction of quinones and quinonoid compounds, with either NADH or NADPH as the electron donor (Iyanagi & Yamazaki, 1970; Iyanagi, 1987). It consists of 2 identical subunits. Each subunit is 273 amino acids long (Robertson et al., 1986; Bayney et al., 1987; Haniu et al., 1988; Jaiswal et al., 1988) and has a molecular weight of 30,000. In addition, each subunit contains 1 FAD prosthetic group that is noncovalently attached to the protein (Hosoda et al., 1974).

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Quinone reductase plays an important role in protecting tissues against the mutagenic, carcinogenic, and cytotoxic effects of quinones that occur widely in nature (Ernster, 1987; Prochaska & Talalay, 1991). Further, evidence has accumulated that the enzyme is involved in reductive activation of the cytotoxic antitumor quinones in cells, such as mitomycins, anthracyclines, and aziridinyl-benzoquinones (Siegel et al., 1990a, 1990b; Boland et al., 1991; Walton et al., 1991). Enzymatic reduction of these anti-tumor quinones gives rise to reactive intermediates that can then undergo nucleophilic additions with DNA and other macromolecules, suggesting a possible mechanism for their cytotoxicity (Lin et al., 1972). Lastly, quinone reductase reduces vitamin K. It can function physiologically as one of several vitamin K reduc-

tases 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 potently inhibited by anticoagulants, such as dicoumarol and warfarin (Hollander & Ernster, 1975; Lind et al., 1979).

Quinone reductase has been purified from 3 sources, rat liver (Ernster et al., 1962; Hosoda et al., 1974; Robertson et al., 1986; Prochaska, 1989; Sharkis & Swenson, 1989), mouse liver (Prochaska & Talalay, 1986), and human adipose tissues (Smith et al., 1988). The rat liver enzyme is the best characterized. Its amino acid sequence has been determined (Haniu et al., 1988) and is in agreement with that deduced from the cDNA sequence (Robertson et al., 1986; Bayney et al., 1987). Extensive structure-function studies of the rat enzyme have been performed by affinity and photoaffinity labeling techniques (Haniu et al., 1988; Liu et al., 1989; Deng et al., 1991) and site-directed mutagenesis experiments (Forrest et al., 1990; Chen et al., 1992; Ma et al., 1992). Site-directed mutagenesis experiments were carried out using either COS cell- or *Escherichia coli*-expression systems. Human quinone reductase has been purified from adipose stromal cells, but it has not been characterized and its primary structure was actually deduced from the cDNA sequence (Jaiswal et al., 1988). The human quinone reductase cDNA was cloned from a human liver cDNA library by Jaiswal et al. (1988) using rat enzyme cDNA as the probe. An additional clone has been obtained by screening the human liver cDNA library and it has a sequence with 40% homology to the quinone reductase clone (Jaiswal et al., 1990). The function of the latter human protein is not yet known.

Quinone reductase was purified from mouse liver in 1986 (Prochaska & Talalay, 1986). Mouse quinone reductase is different from rat and human quinone reductases in 2 respects. First, it was purified in 2 forms, which were characterized as hydrophilic and hydrophobic forms in accordance with their elution from a phenyl-Sepharose CL-4B column with a gradient of glycerol and ethylene glycol (Prochaska & Talalay, 1986). Kinetic studies with dicoumarol established that this compound was a competitive inhibitor with respect to NADH for the hydrophilic form and a mixed-type inhibitor for the hydrophobic form (Prochaska & Talalay, 1986). Second, the purified enzymes were obtained as apoenzyme preparations and the activity could be reconstituted by the addition of FAD. In an effort to determine the differences between the 2 forms, we performed electrospray and tandem (MS/MS) mass spectral analyses to characterize the hydrophilic and the hydrophobic forms of the mouse liver quinone reductase. We also obtained a cDNA clone encoding for mouse quinone reductase by screening a mouse liver cDNA library using the rat quinone reductase cDNA as the probe. Lastly, we expressed mouse quinone reductase in *E. coli* and compared its catalytic properties to those of *E. coli*-expressed rat and human quinone reductase.

Results and discussion

Protein structural analysis of mouse liver NAD(P)H:quinone reductase preparations

Electrospray mass spectral analysis of the hydrophilic and hydrophobic forms of the mouse liver quinone reductase preparations revealed that these 2 forms have identical molecular weights (30,887 and 30,884, respectively; data not shown). The difference in their molecular weights is within the limits of error of such analysis.

Table 1. *Tryptic peptides of the hydrophilic form of mouse liver quinone reductase that were sequenced by tandem mass spectrometry*

HPLC fraction	Avg. nominal $(M+H)^+$	Sequence[a]	Residue nos.
16	1,080	AXXVLAHSEK	5-14
14	962	TSFNYAMK	15-22
10	831	EAAVEAXK	23-30
11	1,220	NDXTGEXKDSK	53-63
13	1,109	XSPDXVAEHK	80-89
7	1,051	SXGHTPPDAR	191-200
18	1,133	MQXXEGWK	201-208
16	1,152	FGXSVGHHXGK	251-261
8	968	SXPADNQXK	262-270

[a] X is either Leu or Ile.

Because only a limited amount of each purified preparation was available, we performed protein structural analysis by using MS/MS technique, as explained in detail in the section below, with the sequence analysis of the amino-terminal peptide as an example. Nine peptides, generated from 50 pmol (1.5 μg) of the hydrophilic form of the mouse enzyme, were sequenced by MS/MS technique. Table 1 indicates the sequence for each of the 9 peptides, the residue numbers (based on the full-length sequence as deduced from the cDNA sequence, see below), HPLC fraction numbers, and average nominal $(M+H)^+$ for each of the peptides. Seven peptides from the same form of the mouse enzyme were sequenced by MS/MS analysis of the chymotrypsin digest (Table 2). A significant number of additional tryptic and chymotryptic peptides were also sequenced by MS/MS analysis, but we were unable to complete the sequence assignment until the cDNA-deduced sequence became available. Ninety percent of the sequence was confirmed by this method, which was important for examining the primary structures of the 2 forms of mouse quinone reductase.

Simultaneous tryptic digestion experiments were performed on both hydrophilic and hydrophobic forms of the mouse qui-

Table 2. *Chymotrypic peptides of the hydrophilic form of mouse liver quinone reductase that were sequenced by tandem mass spectrometry*

HPLC fraction	Avg. nominal $(M+H)^+$	Sequence[a]	Residue nos.
10	700	Ac-AARRAX	1-6
17	1,232	XVXAHSEKTSF	7-17
17	1,033	AMKEAAVEAX	20-29
20	968	EVXESDXY	35-42
28	1,163	AMNFNPXXSR	43-52
25	891	ERVXVAGF	117-124
24	1,089	QVXEPQXVY	182-190

[a] X is either Leu or Ile.

none reductase. The HPLC chromatograms of the 2 forms were very similar, and only differences in yields were observed. No observable differences of mass spectra of the HPLC fractions of the 2 forms were detected. These results, in addition to the identical molecular weights of the 2 forms (as described above), lead us to conclude that the 2 forms of the mouse quinone reductase have the same primary sequence. The difference in the binding affinity of the 2 forms to the phenyl-Sepharose column may result from a posttranslational modification, although we have no evidence to suggest that posttranslational modification occurs. The posttranslational modification(s) has to be a very unstable one because we were not able to detect it during mass spectral analyses. It is also possible that the 2 forms are 2 conformational isomers that exist in different solvent combinations. Additional experiments will be carried out for further evaluation of the difference between 2 forms of the mouse quinone reductase.

Although it has been suggested that quinone reductase might be a glycoprotein (Segura-Aguilar et al., 1992), we did not find any consensus amino acid sequences of glycosylation sites. Moreover, our electrospray mass spectral analyses of quinone reductase preparations from human, rat, and mouse, indicate that this enzyme is not a glycoprotein.

Characterization of the amino-terminal peptide by MS/MS analysis

A chymotryptic peptide located in HPLC fraction 10 has an *m/z* value of 700 for the protonated molecule. A collision-induced dissociation (CID) spectrum for that ion (Fig. 1) yielded sufficient fragmentation to determine the sequence of the peptide and the presence of an acetyl group on the N-terminus. Assignments for the various ions observed in the CID MS/MS spectrum are given in Figure 1. In order to determine for sure that the amino-terminus of this peptide was blocked with an acetyl group, acetylation on the probe was performed. Acetylation was accomplished by addition of 0.5 μL acetic anhydride to the peptide sample, which was run 2 min later. The acetylation procedure would add an acetyl group, or 42 *m/z*, to each free amino group present in a peptide. The mass of a peptide does not change when the peptide is devoid of amino groups or if the amino group is blocked. The acetylation procedure did not change the mass of the chymotryptic peptide, confirming that the amino-terminal alanine residue is blocked with an acetyl group.

This peptide has a sequence homologous with that of the amino-terminus of rat quinone reductase that was also found to be blocked by an acetyl group (Haniu et al., 1988), leading to the hypothesis that this peptide is the amino-terminal peptide of mouse quinone reductase. This was confirmed when the complete amino acid sequence was generated by cloning (see below).

Isolation and characterization of mouse NAD(P)H:quinone acceptor oxidoreductase clone

Using the procedure described under Materials and methods, we obtained 3 positive isolates. The isolate containing the longest

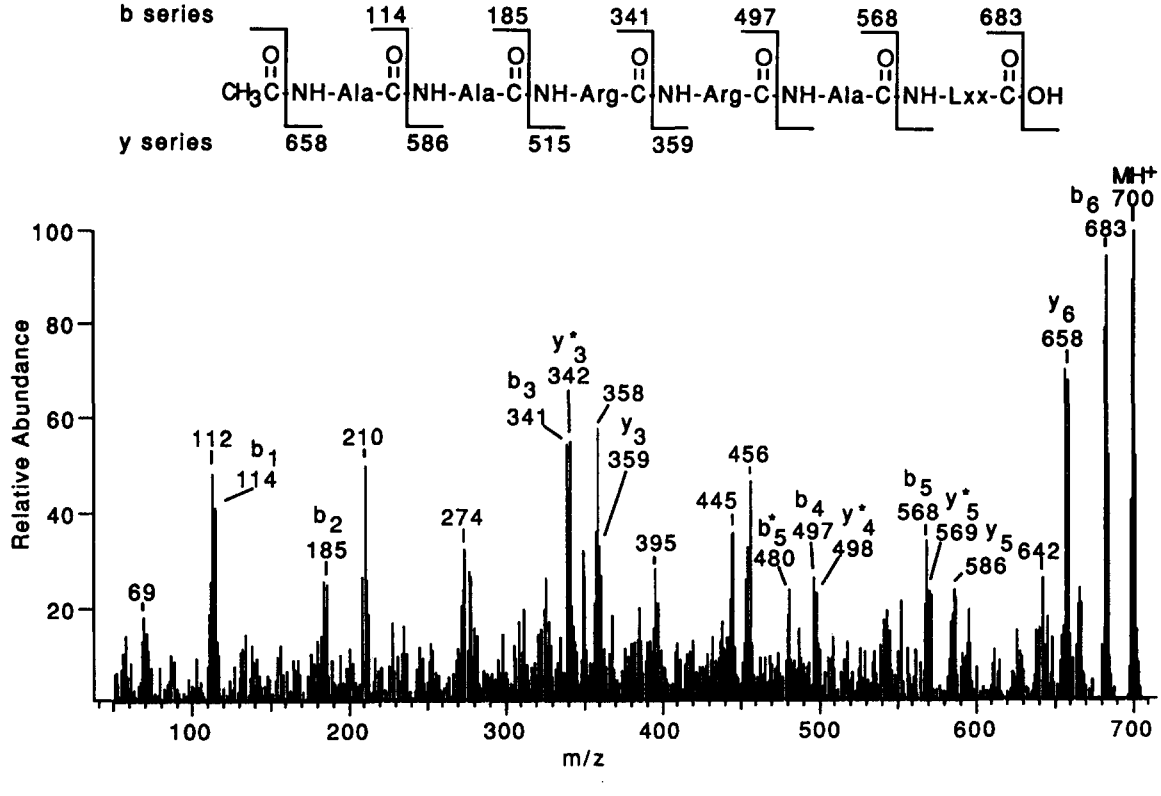

Fig. 1. Positive ion, MS/MS spectrum of the amino-terminal peptide of the mouse quinone reductase. Fragment ions of the protonated molecule (*m/z*) were generated by CID with argon in the center sector of a triple sector quadruple mass spectrometer. The nomenclature of Biemann (1988) is used to designate fragment ion series, and ion series members that have lost ammonia are designated with an asterisk. Lxx designates either Leu or Ile.

insert, pMDT-3, was sequenced (Fig. 2). The insert in pMDT-3 is 1,654 base pairs long and has a poly(A) tail. The deduced amino acid sequence is shown along with the nucleotide sequence. The peptide sequence data (generated by MS/MS analysis) confirmed that this cDNA encodes for the mouse quinone reductase. There was no discrepancy between the amino acid sequences determined by the 2 methods. The cDNA encodes a protein of 273 amino acids (without the first methionine) with a calculated molecular weight of 30,830. Because we know that the amino-terminus is blocked by an acetyl group, the predicted molecular weight of the mouse enzyme is 30,872. As described above, the purified mouse liver quinone reductase has a molecular weight of 30,884 (or 30,887). The difference between the calculated and actual molecular weights, 12–15 *m/z*, could be due to oxidation of a methionine residue in the purified mouse quinone reductase preparations.

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        10         20         30         40         50         60         70         80         90
CGCGGGGCTG GGACTGTACA GTGGGCTGGG CGGGCATAAG CAGGATATAA AGCCTTCGCT CAGCCCATAC CCCAAGGCTC AGCTCTTACT

       100        110        120        130        140        150        160        170        180
AGCCTAGCCT GTAGCCAGCC CTAAGGATCT CTCCGAAGAG CTTTAGGGTC GTCTTGGCAA CCAGCTGCTC AGCCAATCAG CGTTCGGTAT

       190        200        210        220        230        240        250        260        270
TACGATCCTC CCTCAACATC TGGAGCCATG GCGGCGAGAA GAGCCCTGAT TGTACTGGCC CATTCAGAGA AGACATCATT CAACTACGCC
                           M  A  A  R  R  A  L  I  V  L  A  H  S  E  K  T  S  F  N  Y  A

       280        290        300        310        320        330        340        350        360
ATGAAGGAGG CTGCTGTAGA GGCTCTGAAG AAGAGAGGAT GGGAGGTACT CGAATCTGAC CTCTATGCTA TGAACTTCAA CCCCATCATT
 M  K  E   A  A  V  E   A  L  K   K  R  G   W  E  V  L   E  S  D   L  Y  A   M  N  F  N   P  I  I

       370        380        390        400        410        420        430        440        450
TCCAGAAATG ACATCACAGG TGAGCTGAAG GACTCGAAGA ACTTTCAGTA TCCTTCCGAG TCATCTCTAG CATATAAGGA AGGACGCCTG
 S  R  N   D  I  T  G   E  L  K   D  S  K   N  F  Q  Y   P  S  E   S  S  L   A  Y  K  E   G  R  L

       460        470        480        490        500        510        520        530        540
AGCCCAGATA TTGTGGCCGA ACACAAGAAG CTGGAAGCTG CAGACCTGGT GATATTTCAG TTCCCATTGC AGTGGTTTGG GGTGCCAGCC
 S  P  D   I  V  A  E   H  K  K   L  E  A   A  D  L  V   I  F  Q   F  P  L   Q  W  F  G   V  P  A

       550        560        570        580        590        600        610        620        630
ATTCTGAAAG GCTGGTTTGA GAGAGTGCTC GTAGCAGGAT TTGCCTACAC ATATGCTGCC ATGTACGACA ACGGTCCTTT CCAGAATAAG
 I  L  K   G  W  F  E   R  V  L   V  A  G   F  A  Y  T   Y  A  A   M  Y  D   N  G  P  F   Q  N  K

       640        650        660        670        680        690        700        710        720
AAGACCTTGC TTTCTATCAC CACTGGGGGT AGCGGCTCCA TGTACTCTCT TCAGGGTGTC CACGGGGACA TGAACGTCAT TCTCTGGCCG
 K  T  L   L  S  I  T   T  G  G   S  G  S   M  Y  S  L   Q  G  V   H  G  D   M  N  V  I   L  W  P

       730        740        750        760        770        780        790        800        810
ATTCAGAGTG GCATCCTGCG TTTCTGTGGC TTCCAGGTCT TAGAACCTCA ACTGGTTTAC AGCATTGGCC ACACTCCACC AGATGCCCGC
 I  Q  S   G  I  L  R   F  C  G   F  Q  V   L  E  P  Q   L  V  Y   S  I  G   H  T  P  P   D  A  R

       820        830        840        850        860        870        880        890        900
ATGCAGATCC TGGAAGGATG GAAGAAACGT CTGGAAACCG TCTGGGAGGA GACCCCACTC TATTTTGCTC CAAGCAGCCT GTTTGACCTA
 M  Q  I   L  E  G  W   K  K  R   L  E  T   V  W  E  E   T  P  L   Y  F  A   P  S  S  L   F  D  L

       910        920        930        940        950        960        970        980        990
AACTTTCAGG CAGGATTCTT AATGAAAAAG GAAGTTCAAG AGGAGCAGAA GAAGAACAAG TTTGGCCTCT CTGTGGGCCA TCACCTGGGC
 N  F  Q   A  G  F  L   M  K  K   E  V  Q   E  E  Q  K   K  N  K   F  G  L   S  V  G  H   H  L  G

      1000       1010       1020       1030       1040       1050       1060       1070       1080
AAGTCCATTC CAGCTGACAA CCAGATCAAA GCTAGAAAAT AAGGATTTTT TTCCTAACAT ATGTTAGACG CAGCTTTCTT TTTCCCCAGC
 K  S  I   P  A  D  N   Q  I  K   A  R  K   *

      1090       1100       1110       1120       1130       1140       1150       1160       1170
TTGTCTGACT TGCTTGCTTG TCATTTTTTT CCTTTGCTCC ACGAGGATGG GAAAAGGAGT AAGTTTGCTT CATGCTTTTT TTTTTTTTT

      1180       1190       1200       1210       1220       1230       1240       1250       1260
TGATAGTTCT GCCATAACAA CAAAATGAAT GAAGTCAGAT TAGGAGCCTC AGGGCAAGGT GCAGAAGCGA GCTGGAAATA CTCTTCTAGG

      1270       1280       1290       1300       1310       1320       1330       1340       1350
TCATTTATGC AATATTCGCC ATTTTCTTCG GGCTAGTCCC AGTTAGATGG CATCCAGTCC TCCATCAAGA TTCGTTGTCT ATAATTACCT

      1360       1370       1380       1390       1400       1410       1420       1430       1440
CTCTGTGGTT TAGGGCAGAA GGGAATTGCT CAAAGTAAAC AATGGCCGAG GGACTAACTT GTTTAGCAGT TAGCAGTTAG CTAAAGCCTG

      1450       1460       1470       1480       1490       1500       1510       1520       1530
TTTATGATAC ATCCTGGTTT CAATTACTGT GCAGTGACTG ACATGGCGTC CCAGGGGGTT GGCTCTCCAG CTCTTTTCTG TCTGTACACA

      1540       1550       1560       1570       1580       1590       1600       1610       1620
GCACACCCAG GTCCTGGGAA AGGAATTTTA AAACAGATCT CCGTCTCATT CTTTCTATTT CTTTTTTTTT TTAATCGAAA TAAATGAATA

      1630       1640       1650
CATCACACAT CAAAAAAAAA AAAAAAAAAA AAAG
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Fig. 2. Nucleotide sequence of the mouse quinone reductase cDNA. The nucleotide sequence of the mouse quinone reductase cDNA is shown along with the deduced amino acid sequences.

The cDNA encodes 207 bases of 5′-untranslated sequence and 625 bases of 3′-untranslated sequence. In the coding region (822 bases), there are 61 differences between mouse and rat sequences and 98 between mouse and human sequences. Thaete et al. (1991) reported that 1 quinone reductase mRNA species about 2 kb long was identified by Northern blot analysis with RNA isolated from normal mouse lung epithelial cells (NALIA-C1C10 line). This suggests that our clone is missing part of the 5′-untranslated and/or part of the 3′-end poly(A) sequences. The results obtained from our cloning studies and from Northern blot analysis also support that there is only 1 mouse quinone reductase and the 2 forms have identical primary sequences.

Figure 3 shows the amino acid sequence of mouse quinone reductase aligned with those of rat and human quinone reductases. The sequence of the mouse enzyme shows more homology with the rat sequence than with the human sequence. There are 17 and 37 differences between the mouse sequence and the rat and human sequences, respectively.

Expression of mouse NAD(P)H:quinone acceptor oxidoreductase in E. coli

With pMDT-3 as the template, a full-length cDNA, with an *Nco* 1 site at its 5′-end and a *Hind* III site at its 3′-end, was generated by PCR with 2 primers, as described under Materials and methods. The PCR-generated cDNA was sequenced and subcloned into a prokaryotic expression vector, pKK233.2, through the *Nco* 1 and the *Hind* III sites. Active mouse quinone reductase was expressed in *E. coli* strain JM109, which was transformed with the expression plasmid. The mouse enzyme was purified as described for rat quinone reductase (Chen et al., 1992). The purified mouse enzyme preparations showed a band that migrated faster than the rat enzyme on SDS polyacrylamide gel electrophoresis (Fig. 4). The accurate molecular weight of the *E. coli*-expressed mouse enzyme was determined to be 30,835 by electrospray mass spectrometry and was very similar to the molecular weight calculated from the sequence, 30,830. This result indicates that the amino-terminus of the *E. coli*-expressed mouse quinone reductase is not acetylated, resembling the rat quinone reductase expressed in *E. coli* (Chen et al., 1992).

Characterization of E. coli-expressed mouse NAD(P)H:quinone acceptor oxidoreductase

The *E. coli*-expressed mouse quinone reductase was purified as the holoenzyme containing the FAD prosthetic group, whereas enzyme purified from mouse liver does not contain FAD (Prochaska & Talalay, 1986). The reason for this difference in FAD binding of the 2 preparations is not understood. Because the enzyme was purified from mouse liver by a procedure that is very different from the one used here for the purification of the *E. coli*-expression enzyme, it is possible that the FAD prosthetic group was dissociated from the enzyme during the previous purification procedure used for the liver enzyme.

The *E. coli*-expressed mouse quinone reductase has a higher NAD(P)H-menadione reductase activity than the *E. coli*-expressed rat and human quinone reductase. As shown in Table 3, the *E. coli*-expressed mouse quinone reductase was at least twice as active as the *E. coli*-expressed rat and human quinone reductase. The K_m values of NADPH and NADH for the mouse enzyme were found to be similar to those of the human enzyme and slightly higher than the values for the rat enzyme. Furthermore, the K_m value of menadione for the mouse enzyme was found to be slightly higher than those for the rat and human enzymes. Even though the assay procedures were different, the kinetic values of the *E. coli*-expressed mouse quinone reductase were not much different from those for the purified mouse liver enzyme preparations (Prochaska & Talalay, 1986). In addition, the *E. coli*-expressed mouse quinone reductase is inhibited by dicoumarol in a competitive manner with respect to NADH (results not shown).

In addition to a higher NAD(P)H-menadione reductase activity, mouse quinone reductase was also found to be significantly less sensitive to flavones and dicoumarol than the rat and human enzymes. Flavones have been found to be potent inhibitors of the quinone reductase and have been suggested to be potentially useful oral anticoagulants (Liu et al., 1990; Chen et al., 1993). These compounds inhibit quinone reductase in a competitive manner with respect to NAD(P)H. As shown in Figure 5A, chrysin, 5,7-dihydroxyflavone, is a very poor inhibitor of mouse quinone reductase. Similar results were obtained when other fla-

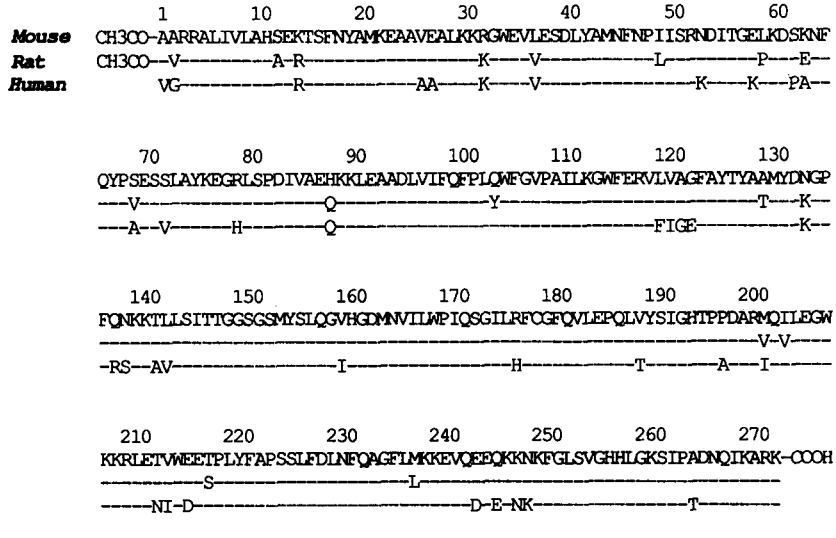

Fig. 3. Amino acid sequences of NAD(P)H:quinone acceptor oxidoreductase from rat, human, and mouse liver. For the amino acid sequences of the human and mouse enzyme, only the residues that are different from those of the rat enzyme are shown.

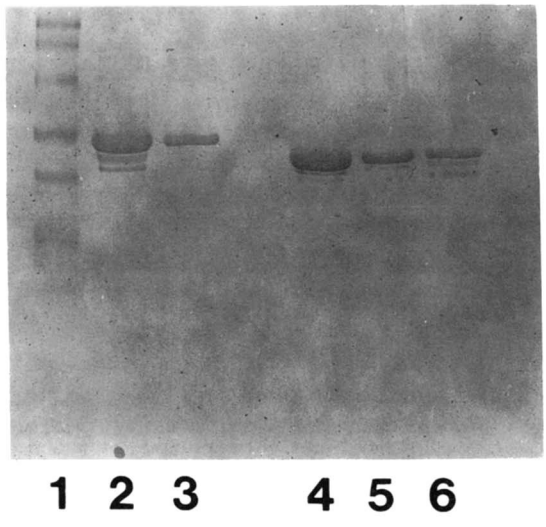

Fig. 4. SDS-PAGE of mouse and rat NAD(P)H:quinone acceptor oxidoreductase. The purified enzyme preparations were examined on an SDS/12% polyacrylamide gel. Lane 1, the molecular weights of molecular weight markers (Std) are 97,400, 66,200, 45,000, 31,000, 21,500, and 14,000; lane 2, the *E. coli*-expressed rat quinone reductase (9 μg); lane 3, purified rat liver quinone reductase (3 μg); lane 4, the *E. coli*-expressed mouse quinone reductase (9 μg); lane 5, purified mouse liver quinone reductase, the hydrophilic form (3 μg); and lane 6, purified mouse liver quinone reductase, the hydrophobic form (3 μg). The minor bands shown on the gel are proteolytic products of the enzyme. As indicated in a recent publication (Chen et al., 1994), the carboxyl-terminal portion of the enzyme is very sensitive to proteolysis.

vones were used (results not shown). Dicoumarol, an oral anticoagulant and a very potent inhibitor of quinone reductase, was also found to be a less effective inhibitor of mouse quinone reductase in comparison to the rat and human enzymes (Fig. 5B). As indicated in the introduction, NAD(P)H:quinone acceptor oxidoreductase may be 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 and is the target of anticoagulants, such as dicoumarol and flavones. However, the binding mechanisms of these compounds to the quinone reductase are not yet known. The present finding would suggest that the regions in the mouse quinone reductase that contain amino acids (such as Lys-62, Ser-68, and Met-200) different from the rat and human enzymes, are the critical regions involved in the binding of the above-mentioned compounds. Therefore, the availability of the amino acid sequences and the inhibition profiles of the quinone reductase of the 3 species by dicoumarol and flavones would allow one to design site-directed mutagenesis experiments to characterize the binding mechanisms of these compounds. Such studies have been initiated in our laboratory.

In summary, in this study we determined the primary structure of the mouse quinone reductase and expressed the enzyme in *E. coli*. From mass spectral and molecular cloning studies, we concluded that the 2 forms of the mouse enzyme have identical primary sequences. In addition, although the enzyme was purified from mouse livers in an apoenzyme, without FAD, the *E. coli*-expressed enzyme had bound FAD. Differences in the purification procedures may account for this discrepancy. Lastly, our results have revealed that the amino acid sequence information for the mouse quinone reductase is very important in designing site-specific mutants for a characterization of the binding mechanism of oral anticoagulants.

Materials and methods

Materials

NAD(P)H, menadione, dicoumarol, trypsin, and chymotrypsin were purchased from Sigma Co. The proteases were purified through reverse-phase HPLC before use.

T4 kinase, T4 DNA ligase, and restriction endonucleases were obtained from Boehringer Mannheim Biochemicals, New England Biolabs, Inc., and Bethesda Research Laboratories. Taq DNA polymerase was from Perkin Elmer. The random primer labeling kit was purchased from Boehringer Mannheim. The Sequenase Version 2.0 DNA Sequencing System was from United States Biochemical Co. The *E. coli* expression plasmid pKK233.2 was from Pharmacia Co. The mouse liver cDNA Lambda Zap™ library was purchased from Stratagene Co. Radiolabeled nucleotides were from New England Nuclear. Oligonucleotide prim-

Table 3. *Catalytic properties of E. coli-expressed mouse, rat, and human NAD(P)H:quinone acceptor oxidoreductase*

	NADPH[a]		NADH		Menadione	
	K_m[b]	V_{max}	K_m	V_{max}	K_m	V_{max}
Mouse	170(±40)[c]	19,000(±3,600)	320(±120)	21,000(±6,200)	4.1(±0.7)	16,900(±1,000)
Rat	120(±10)	8,700(±900)	190(±60)	8,500(±1,900)	2.6(±0.6)	6,600(±800)
Human[d]	240(±60)	8,500(±1,400)	290(±100)	7,300(±2,000)	2.0(±1.0)	8,700(±2,800)

[a] The NAD(P)H-menadione reductase activity of these preparations was determined spectrophotometrically by measuring the reduction of MTT at 610 nm at 25 °C. The assay mixture (1 mL) contained 50 mM potassium phosphate, pH 7.5, 200 μM NAD(P)H, 10 μM menadione, and 0.3 mg/mL of MTT. In this assay, menadione is the electron acceptor and MTT is included in order to continuously reoxidize the menadiol formed. For determination of kinetic values for NAD(P)H, the NAD(P)H concentration was varied. For determination of kinetic values for menadione, the menadione concentration was varied.

[b] The unit for K_m is μM and the unit for V_{max} is μmol/mg/min.

[c] The analysis was performed 3 times.

[d] The human quinone reductase was purified from *E. coli* transformed by an expression plasmid that was kindly provided by Dr. N. Gibson and his colleagues at the University of Southern California, Los Angeles.

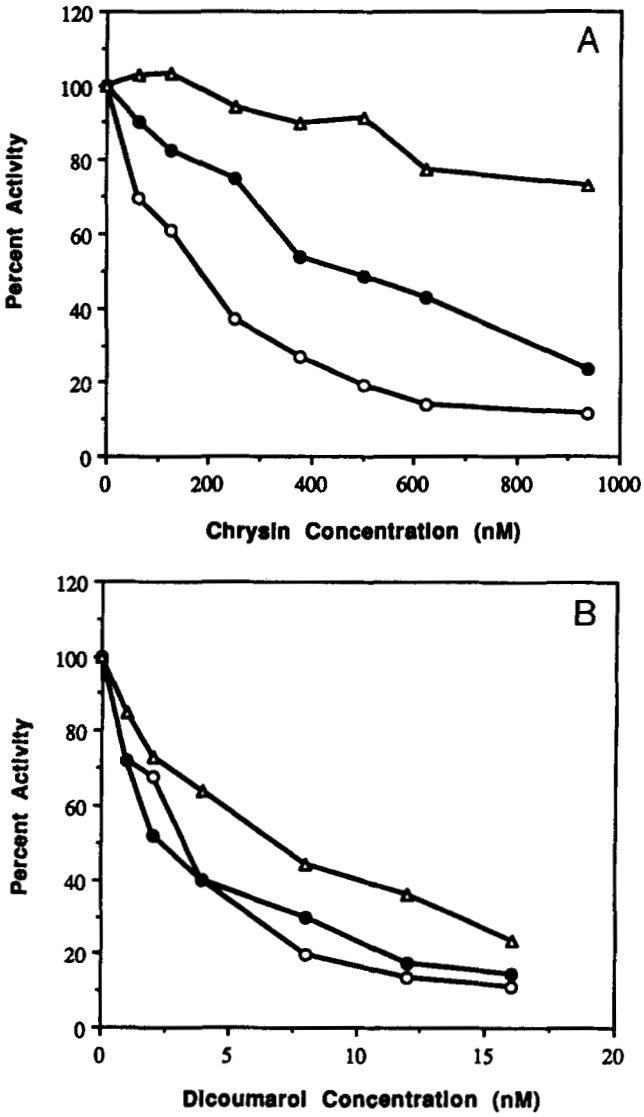

Fig. 5. Inhibition of NAD(P)H:quinone acceptor oxidoreductase of the 3 species by chrysin (**A**) or dicoumarol (**B**). The inhibition study was performed with quinone reductase of the 3 species at amounts providing an identical level of the NADH-menadione reductase activity. The assay conditions are those provided in the Materials and methods. The experiment was repeated twice. All of the assays were initiated with the addition of the enzyme and were performed in duplicate. △, The mouse quinone reductase; ●, the rat quinone reductase; ○, the human quinone reductase.

ers used for this study were synthesized at the DNA synthesis facility at the City of Hope.

Protein sequence analysis

Proteolytic digestion of mouse liver NAD(P)H:quinone acceptor oxidoreductase

Approximately 1.5 μg of the previously purified mouse enzymes were each dissolved in 50 μL of 90% ammonium bicarbonate buffer (50 mM) and 10% acetonitrile (sequence grade). HPLC-purified trypsin or chymotrypsin was then added to each solution in a protease to quinone reductase ratio of 1:100 (w/w). The digestions were carried out at room temperature in a microcentrifuge rotating at 500 rpm for 12 h.

Peptide purification by reverse-phase HPLC

HPLC separation of peptides was performed with a micro-HPLC system designed and constructed at the Beckman Research Institute of the City of Hope (Davis & Lee, 1992). Proteolyzed samples (20 μL) were injected onto a 0.5 × 25-cm fused silica capillary column packed with a reverse-phase C18 column packing (Vydac Co., Hesperia, California). Columns were eluted with a linear gradient of 2 solvents (A and B) for a period of 30 min. Solvent A was 0.1% trifluoroacetic acid (TFA) in deionized water. Solvent B was 90% acetonitrile, 10% deionized water, and 0.07% TFA. Solvent B increased from 1% to 80% during the 30-min period. Absorbance of the column effluent was monitored at 214 nm. Fraction volumes ranged from 5 to 10 μL.

Mass spectrometry

Mass spectra were obtained with a Finnigan MAT TSQ-700 triple sector quadruple mass spectrometer (Finnigan-MAT, San Jose, California) equipped with a cesium ion gun (Phrasor Scientific, Duarte, California). The primary ion beam energy was 6 keV. Samples were prepared for analysis by adding 0.5 μL of a previously collected HPLC fraction directly to a stainless steel sample stage coated with 0.5 μL of 1-thioglycerol. Both normal MS and CID MS/MS spectra were collected in an automated procedure by using a program written in instrument control language (ICL). A single MS scan ranging from 400 to 2,000 *m/z* was collected. The instrument then automatically switched to the MS/MS mode and 10 CID spectra for the most intense ion observed in the original MS spectrum were collected. Analysis of HPLC fractions containing mixtures of peptides required additional sample runs with manual selection of the precursor ion for the CID MS/MS spectra.

Electrospray mass spectra of the intact mouse quinone reductase preparations were also obtained on a Finnigan MAT TSQ-700 mass spectrometer equipped with an electrospray ion source as described previously (Chen et al., 1992; Swiderek et al., 1992). Briefly, the enzyme preparations were loaded on to a microcapillary HPLC system interfaced with the Finnigan instrument. Scans were acquired every 3 s in a mass range from 500 to 2,000. Spectra were generated by averaging the scans containing the peak and the mass assignments were made by using the Finnigan MAT BIOMASS data reduction software.

Analysis of HPLC fractions containing mixtures of peptides required additional sample runs in a manual mode of instrument operation.

Isolation of mouse NAD(P)H:quinone acceptor oxidoreductase cDNA clone

cDNA library screening

The mouse cDNA library was screened with procedures described by Sambrook et al. (1989) using a ^{32}P-labeled rat quinone reductase cDNA probe. About 5×10^5 plaque-forming units (PFUs) were plated in *E. coli* stain SURE (Stratagene Co.). Plaques were blotted onto nylon filters (Micron Separations Inc., Westboro, Massachusetts), denatured with alkali, neutral-

ized, and baked. The filters were hybridized with 1.2×10^8 cpm ^{32}P-labeled rat quinone reductase probe in 6× standard saline citrate (SSC) and 0.05× Blotto at 58 °C for 42 h. Filters were washed in 2× SSC containing 0.1% SDS at 58 °C for 1 h and then exposed to X-OMAT™ films (Eastman Kodak Co., Rochester, New York). Three positive isolates were plated to homogeneity and then excised from the lambda ZAP™ plasmid and isolated as pBluescript™ constructs as described by the manufacturers. One of these 3 isolates was found to contain an insert of approximately 1,600 bp. This isolate was designated pMDT-3 and sequenced.

DNA sequencing

DNA sequences were determined by the Sanger dideoxy sequencing method using the Sequenase Version 2.0 Sequencing System. DNA sequencing was done in 2 stages. Initially, *Sau* 3A- and *Hae* III-digested fragments from pMDT-3 were ligated into the *Bam* H1 or *Sma* 1 sites of pUC19, respectively. These subclones were partially sequenced with either M13/PYC -47 or reverse plasmid primers (New England Biolab.) to obtain a partial sequence. Oligonucleotides with the quinone reductase sequences were then synthesized and used to fill in gaps in the sequence.

Expression of the mouse NAD(P)H:quinone acceptor oxidoreductase in E. coli

Generation of the mouse quinone reductase cDNA by polymerase chain reaction (PCR)

PCR was carried out under standard conditions. Briefly, reaction volumes were 100 μL. Samples were denatured at 94 °C for 30 s, annealed at 55 °C for 30 s, and extended at 72 °C for 1 min. The reaction was carried out for 30 cycles with 0.5 μg each of 2 oligonucleotide primers (i.e., an amino-terminal primer containing an *Nco* 1 restriction site with nucleotide sequence encoding for the amino-terminal 7 amino acids, 5′-GCTCATGAAC TACTCTGGGGGCTGCTACACGGCCATGGCFGCGAGAA GAGCCCTG-3′ and a carboxyl-terminal primer containing a *Hind* III restriction site with nucleotide sequence encoding for the carboxyl-terminal 6 amino acids, 5′-AGCAGAGAGCATAA GAACAGAAAGCTTATTTTCTAGCTTTGATCTG-3′ with 1 μg of pMDT-3 cDNA as template.

Cloning of the PCR product into the expression plasmid pKK233.2

The PCR-amplified DNA (1 kb in size) was separated and extracted from an agarose gel and cloned into the TA™ vector (Invitrogen Co., San Diego, California). The mouse quinone reductase cDNA was isolated by restricting the TA™ plasmid with restriction enzymes *Nco* 1 and *Hind* III, and ligated into the pKK233.2 plasmid through the *Nco* 1 and *Hind* III restriction sites. This plasmid was designated pL30.1.

E. coli expression

Conditions for expressing the mouse quinone reductase in *E. coli* are identical to those used for the expression of rat quinone reductase (Chen et al., 1992). Briefly, the mouse enzyme expression plasmid was transformed into the JM109 strain. Two milliliters of 2× YT (1 L of 2× YT contains 16 g of Bactotryptone, 10 g of yeast extract, and 10 g of NaCl, pH 7.5) containing ampicillin (75 μg/mL) were inoculated with a single colony of the JM109-mouse quinone reductase-pKK transformant. After overnight incubation at 37 °C, the 2-mL culture was used to inoculate 50 mL of prewarmed 2× YT. When the absorbance of the *E. coli* culture at 600 nm reached 0.6, isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 2 mM to induce expression of the quinone reductase. Five-milliliter aliquots were withdrawn at different time intervals, and the level of quinone reductase activity was determined.

Purification of the expressed quinone reductase

Two liters of transformed JM109 cultures were grown for 17 h in the presence of 2 mM IPTG and harvested as described in the previous section. The cells were washed with 200 mL of phosphate-buffered saline (PBS), followed by 200 mL of buffer A (50 mM Tris buffer, pH 7.5, and 100 mM NaCl), and resuspended in 60 mL of buffer A. After sonication (3×20 s at 40% duty cycle with output control setting at 4), the cell debris was removed by centrifugation at 10,000 rpm ($13,800 \times g$) for 10 min using a Beckman JA-17 rotor. The pellets were resuspended in 60 mL of buffer A and sonicated again. The supernatant fractions were combined and centrifuged at 40,000 rpm ($105,000 \times g$) for 90 min with a Beckman 50.2Ti rotor. The supernatant fraction was applied to a 50-mL Affi-gel Blue (Bio-Rad) column (2.5×10 cm), which was equilibrated with 500 mL of buffer A. The elution procedure was essentially the same as described by Prochaska (1989) except that 50% ethylene glycol was omitted in the final elution buffer and the enzyme was not released from the Affi-gel Blue resin by washing with 20 mM Tris buffer, pH 10, containing 1 mM NADH. Instead, the column was washed with 3 bed volumes of 50 mM Tris buffer, pH 7.5, 0.25 M sucrose, and 0.5 M KCl as described by Sharkis and Swenson (1989). The enzyme was eluted from the column by using the latter buffer containing 8 mM NADH. The active fractions were pooled and concentrated by precipitation with 75% ammonium sulfate. The enzyme pellet was resuspended in a small volume of 50 mM potassium phosphate, pH 7.5, and dialyzed against the same buffer. The purified enzyme was stored at −70 °C. Eight milligrams of the enzyme were purified from a 2-L culture.

Enzyme assay

The NADH-menadione reductase activity of the enzyme was determined spectrophotometrically by measuring the reduction of 3-(4,5-dimethylthiazo-2-yl)2,5-diphenyltetrazolium bromide (MTT) at 610 nm as described by Chen et al. (1993) at 25 °C. The assay mixture (1 mL) contained 50 mM potassium phosphate, pH 7.5, 200 μM NADH, 10 μM menadione, and 0.3 mg/mL of MTT. In this assay, menadione is the electron acceptor, and MTT is included for continuous reoxidation of the menadiol formed.

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