Unexpected genetic and structural relationships of a long-forgotten flavoenzyme to NAD(P)H:quinone reductase (DT-diaphorase)

(nonphosphorylated dihydronicotinamides/hydride transfer/flavoprotein/polycyclic aromatic hydrocarbons)

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ABSTRACT A mammalian cytosolic FAD-dependent enzyme that catalyzes the reduction of quinones by *N*-ribosyl- and *N*-alkyldihydronicotinamides, but not by NADH, NADPH, or NMNH (reduced nicotinamide mononucleotide), was isolated from bovine kidney more than 30 years ago [S. Liao, J. T. Dulaney and H. G. Williams-Ashman (1962) *J. Biol. Chem.* **237**, 2981–2987]. This enzyme is designated here as quinone reductase type 2 (QR_2). Bovine QR_2 is a homodimer that migrates on SDS/PAGE at ≈ 22 kDa. Three tryptic peptides of bovine QR_2 (representing 39 amino acids) showed 43% identity to human NAD(P)H:quinone reductase (DT-diaphorase; EC 1.6.99.2), here designated QR_1 and 82% identity to a related human cDNA clone [called $hNQO_2$ by A. K. Jaiswal, P. Burnett, M. Adesnik and O. W. McBride (1990) *Biochemistry* **29**, 1899–1906], and designated here as hQR_2. The protein encoded by the latter cDNA did not show QR activity when tested with conventional nicotinamide nucleotides. The unexpected high homology between the old flavoenzyme and hQR_2 prompted us to clone and overexpress hQR_2. The properties of hQR_2 were identical to those of the flavoenzyme described by S. Liao and H. G. Williams-Ashman, thus establishing their genetic identity. Recombinant human QR_2: (*i*) reacts with *N*-ribosyl- and *N*-alkyldihydronicotinamides, but not with NADH, NADPH, or NMNH; (*ii*) is very weakly inhibited by dicumarol or Cibacron blue; (*iii*) is very potently inhibited by benzo[*a*]pyrene. The x-ray crystal structure of rat QR_1 shows that the 43 amino acid C-terminal tail of QR_1 provides the binding site for the hydrophilic portions of NADH and NADPH. In the absence of this binding site in QR_2, the enzyme retains the essential catalytic machinery, including affinity for FAD, but cannot bind phosphorylated hydride donors.

In the early 1960s Liao and Williams-Ashman (1–4) described the purification and properties of a previously unknown mammalian cytosolic FAD-dependent flavoprotein that catalyzes the oxidation of reduced *N*-ribosyl- and *N*-alkylnicotinamides by menadione and other quinones (Scheme **I**), but not by NADH, NADPH, and NMNH.

Although this enzyme was highly purified from bovine kidney, and its kinetics, substrate, and inhibitor specificities were determined (4), it has been completely ignored for more than 3 decades, and no information on its structure is available. For reasons to be explained, we here designate this flavoprotein as quinone reductase type 2 (QR_2).† Liao *et al.* (4) pointed out that QR_2 has substrate and inhibitor specificities that are strikingly different from those of the more widely known NAD(P)H:(quinone acceptor) oxidoreductase or DT- diaphorase (EC 1.6.99.2)‡

R = Ribosyl, Alkyl or Phenyl
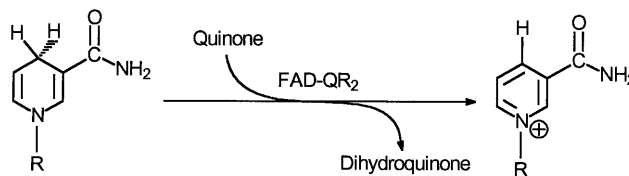
Scheme **I**

(here designated QR type 1 or QR_1). For example, (*i*) QR_2 does not react with NADH, NADPH, or NMNH, all of which are hydride donors for QR_1; (*ii*) QR_2 is very weakly inhibited by dicumarol, whereas QR_1 is exquisitely sensitive to this anticoagulant; (*iii*) QR_2 is potently inhibited by polycyclic aromatic hydrocarbons and by the estrogens 17β-estradiol, equilenin, and diethylstilbestrol, which only weakly inhibit QR_1; and (*iv*) QR_2 is only modestly, if at all, inducible, whereas QR_1 is induced by a wide variety of structurally distinct compounds (6, 7). Despite these differences, we show in this paper that QR_1 and QR_2 are closely related in sequence and structure.

Our laboratory has had a long-standing interest in QR_1 because this enzyme is highly inducible by a variety of Michael acceptors and other electrophiles, and its induction is associated with the protection of animals and their cells against electrophile toxicity and chemical carcinogenesis (8). By virtue of its unusual and obligatory 2-electron reduction mechanism (9), QR_1 diverts quinones from participating in oxidative

cycling that can generate reactive oxygen species and from depleting intracellular glutathione (8). QR_1 is also involved in the reductive activation of certain chemotherapeutic quinones such as mitomycins and aziridylbenzoquinones (10). The enzyme is therefore of dual importance for cancer research: it plays roles in both chemical protection against cancer and in the bioactivation of certain cancer chemotherapeutic agents.

The recent report by Friedlos *et al.* (11) that QR_1 could use simple reduced quaternary nicotinamides, such as *N*-methyldihydronicotinamide and *N*-ribosyldihydronicotinamide, as hydride donors in place of NADH or NADPH, raised the question whether QR_1 and QR_2 might be structurally and possibly even functionally related. We examined this possibility in the hope that the three-dimensional crystal structure of rat QR_1 (rQR_1), recently determined by x-ray diffraction to 2.1-Å resolution (12), might explain the differences in reactivity of the two enzymes with various substituted dihydronicotinamides.

Therefore, we purified bQR_2 from bovine kidney by modern improvements of the procedure of Liao *et al.* (4). We then sequenced three tryptic peptides obtained from bQR_2 (consisting of 8, 11, and 20 amino acids) and found that they were highly homologous (82% identical) to the sequence of a cloned human cDNA isolated by Jaiswal and colleagues (5, 13) and designated by these authors as $hNQO_2$. Jaiswal *et al.* (5, 13) serendipitously isolated the cDNA for $hNQO_2$ during the cloning of human QR_1 (hQR_1). The deduced amino acid sequences of the two cDNAs coding for hQR_1 and $hNQO_2$ were very similar, but no known function was assigned to $hNQO_2$.

The unexpected high homology (82% identity) of the peptides of bQR_2 with the amino acid sequence deduced from the human cDNA clone of Jaiswal *et al.* (5) suggested to us that these enzymes might be genetically identical. Therefore, we cloned the cDNA for $hNQO_2$ and overexpressed and purified the protein that it encodes. This purified protein (which we designate here as QR_2) has enzymatic properties identical to those of the flavoprotein isolated by Liao and Williams-Ashman 30 years ago (1–4). The homodimeric subunits of QR_2 comprise 231 amino acids, whereas those of QR_1 contain 274 amino acids.[§] The amino acid sequences of QR_1 and QR_2 can be aligned without insertions or deletions, but QR_2 lacks 43 of the C-terminal residues. Examination of the x-ray crystal structure of rQR_1 (12) clarifies the reasons for the differences between the electron donor specificities of QR_2 and QR_1, since the 43 amino acid carboxyl tail of QR_1, which is absent in QR_2, provides the binding site for the pyrophosphate–ribose–adenine moiety of NADH and NADPH. This finding also explains the recent observation that QR_1 can use not only NADH, NADPH, and NMNH as hydride donors, but also a variety of synthetic reduced *N*-alkylnicotinamides (11).

EXPERIMENTAL PROCEDURES

Materials. Protein sequence grade trifluoroacetic acid (TFA) (25%), ammonium sulfate, NADH, NADPH, NMNH, and buffer salts were purchased from Sigma. *N*-Methylnicotinamide iodide (3-carbamoyl-1-methylpyridinium iodide), sodium hydrosulfite, menadione (vitamin K_3), benzo[*a*]pyrene, and dicumarol were obtained from Aldrich. Cibacron blue was crystallized as the choline salt from methanol (14). All solvents were obtained from J.T. Baker. Centriprep-10 cartridges were from Amicon. Phenyl-Sepharose 6 (fast flow; high substitution), Q-Sepharose Fast Flow, Mono-Q ion exchange column, Superose-12 HR 10/30 gel filtration column, and the fast protein liquid chromatography apparatus were from Pharmacia. The synthesis of oligonucleotides and automatic sequencing of plasmid DNAs were carried out at the Genetic Core Facility of The Johns Hopkins University School of Medicine.

Preparation of *N*-Methyldihydronicotinamide. *N*-Methyldihydronicotinamide was prepared from *N*-methylnicotinamide iodide according to Rafter and Colowick (15) and stored in 10 mM sodium bicarbonate buffer at pH 10.5 (16). The concentrations of *N*-methyldihydronicotinamide solutions were determined by use of the molar absorption coefficient (ε_m) of 7000 $M^{-1}\cdot cm^{-1}$ at 360 nm (15).

Enzyme Assays. Enzyme activities were determined spectrophotometrically at 25°C, in cuvettes of 1.0-cm light path containing in final volumes of 3.0 ml or 1.0 ml: 60 μM menadione, 80 μM *N*-methyldihydronicotinamide, 50 mM Tris·HCl buffer (pH 8.50), 0.1% (vol/vol) Triton X-100, and 100 μM dicumarol. The menadione was added as a 30 mM stock solution in ethanol, and the *N*-methyldihydronicotinamide was added as an 80 mM stock solution (pH 10.5). The absorbance of the reaction mixture was monitored at 360 nm. Minor nonenzymatic decreases in absorbance at 360 nm were subtracted from the total rates to yield the initial enzymatic reaction velocities. The specific activity of the enzyme was calculated on the basis of molar absorbance coefficients (ε_m) of 7000 $M^{-1}\cdot cm^{-1}$ for *N*-methyldihydronicotinamide and 1000 $M^{-1}\cdot cm^{-1}$ for menadione at 360 nm. The oxidized form of *N*-methylnicotinamide and reduced menadione do not absorb significantly at 360 nm. Specific activities were expressed with respect to protein concentrations measured by the method of Bradford (17) with crystalline BSA as standard.

Purification of QR_2 from Bovine Kidney. The procedures were modified from those described by Liao *et al.* (4). Bovine kidneys were obtained from the slaughterhouse and quickly frozen. All subsequent operations were carried out at 4°C, unless otherwise noted. One bovine kidney was dissected free from fat and connective tissues and chopped into small pieces. The tissue (420 g) was homogenized in 1 liter of 0.25 M sucrose/3 mM sodium bicarbonate solution. The homogenate was centrifuged at 16,300 × g for 40 min. The supernatant fluid was fractionated with ammonium sulfate, and the fraction precipitating between 40 and 70% saturation was collected, dissolved in 190 ml of 20 mM sodium phosphate buffer (pH 6.8), and dialyzed against water for 24 h. Then, solid KH_2PO_4 was added to a final concentration of 0.1 M and the pH was adjusted to 4.2 with acetic acid. This solution was heated for 10 min at 38°C to denature most of the QR_1 activity, while minimizing the loss of the desired QR_2 activity (4). The partially purified material was precipitated with 70% saturated ammonium sulfate, redissolved, and loaded onto a phenyl-Sepharose column in 10 mM Tris acetate (pH 7.5), containing 500 mM sodium chloride, and eluted with a linear gradient from 100% 10 mM Tris acetate/500 mM sodium chloride (pH 7.5) to 100% of a mixture of equal volumes 10 mM Tris acetate (pH 7.5) and ethanol. After concentration of the pooled active fractions (Centriprep-10 cartridges) and dialysis against 5 mM Tris acetate buffer (pH 7.5), the solution was loaded onto a Q-Sepharose Fast Flow column and eluted with a linear gradient from 100% 5 mM Tris acetate (pH 7.5) to 100% 5 mM sodium phosphate/200 mM sodium chloride (pH 6.0). The fractions containing the desired activity were pooled and concentrated by centrifugation on Centriprep-10 cartridges. The proteins were rechromatographed on smaller phenyl-Sepharose and Mono-Q columns, under conditions similar to those described above. Gel filtration was then carried out on a Superose-12 column (10 × 300 mm) in 5 mM sodium phosphate (pH 6.5), containing 200 mM sodium chloride. The column was calibrated with molecular weight markers: BSA (66 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa).

Homogeneity of the Purified bQR_2 and Its Amino Acid Sequence. Purified bQR_2 was subjected to SDS/PAGE on 17% gels according to Laemmli (18). A Waters HPLC system and a Waters Delta Pak 5-μm C_4 column (3.9 × 150 mm) were used to analyze the protein and prepare the sample for tryptic digestion and protein sequencing. Two solvent systems were used: (A) 0.1% TFA in water and (B) 0.1% TFA in 85%

[§]Both rQR_1 and hQR_1 contain 274 amino acids according to their cDNA sequence. The purified rQR_1, however, contains 273 amino-acids; the N-terminal methionine residue is absent (see ref. 12).

acetonitrile in water (19). The enzyme was eluted (2.0 ml/min) with a linear gradient from 50% A: 50% B to 30% A: 70% B over a 40-min period at 25°C, monitored at 215 nm. Protein sequencing, amino acid analysis, tryptic digestion, and HPLC separation of peptides, matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) (20) of peptides, and Edman sequencing were carried out at the Harvard Micro-Chem Facility (Cambridge, MA).

Cloning of hQR$_2$ Gene. Total cellular RNA was isolated from normal human liver by use of guanidine isothiocyanate-sodium sarkosyl-2-mercaptoethanol, centrifuged through cesium chloride (21), and quantitated by UV absorption. The human liver RNA was reverse transcribed with oligo(dT). The reaction mixture (20 μl) contained: 2 μg RNA, 0.5 μM oligo(dT), 0.5 mM dNTP mixture, 62.5 mM Tris·HCl (pH 8.3), 11.5 mM DTT, 11.25 mM MgCl$_2$, and 12.5 units of reverse transcriptase (avian myeloblastosis virus). The cDNA was then amplified for 30 cycles of 95°C for 1 min, 65°C for 1 min, and 72°C for 1 min in a thermocycler (Ericomp, San Diego), and followed by a final 5-min extension at 72°C. The PCR mixture (50 μl) contained 2 μl of reverse transcriptase cDNA product, 10 mM Tris·HCl (pH 8.3), 1.5 mM MgCl$_2$, 50 mM KCl, 0.2 mM dNTP mixture, 0.2 μM of sense and antisense primers corresponding to the N terminus (primer 1) and the C terminus (primer 2) of the 693 bp open reading frame of the reported cDNA sequence (5), and 2.5 units of *Taq* DNA polymerase (Boehringer Mannheim).

The primers had the following sequences: Primer 1: 5′-GCATATGGCAGGTAAGAAAGTACTC-3′ (sequence for an added *Nde*I site and the initiation of translation is underlined) and Primer 2: 5′-CTATTATTGCCCGAAGTGCCAGTG-3′ (antisense sequence of two stop codons TAA and TAG is underlined).

After excision of the ≈700-bp band obtained from reverse transcription–PCR from a low melting point agarose gel, the fragment was ligated into the TA cloning vector (Invitrogen). Colonies with inserts were selected by plating *Escherichia coli* DH5α cells, which had been transformed with the ligation mixture on an LB (Luria broth) plate containing ampicillin and 5-bromo-4-chloro-3-indolyl β-D-galactoside. Over 90% of the white colonies contained the desired insert. The plasmid DNA was isolated by use of a Qiagen (Chatsworth, CA) Miniprep Kit, and restriction analysis of the fragment with *Nde*I, *Eco*RI, and *Bam*HI identified the inserts in the desired orientation. The cDNA coding for the QR$_2$ protein has a single *Bam*HI restriction site. The complete sequence of the insert from both strands was determined by automatic sequencing and was found to be identical with that reported by Jaiswal *et al.* (5). The hQR$_2$ gene was cut by *Nde*I/*Eco*RI digestions and then ligated into a pET-22 expression vector (Novagen), which had been linearized by *Nde*I/*Eco*RI digestions. The ligation mixture was then used to transform *E. coli* DH5α. PCR with primers 1 and 2 of hQR$_2$, with the colonies dispersed in a small amount of LB solution as templates, showed that 9 of 10 colonies carried the desired transformation of the hQR$_2$ gene. The pET-22 vector with hQR$_2$ insert was then transformed into *E. coli* BL21(DE3) pLys S or BL21(DE3) (Novagen) for overexpression after Miniprep of DNA from the DH5α culture.

Overexpression of Recombinant hQR$_2$ and Its Catalytic Activity. The *E. coli* strain carrying the hQR$_2$ gene was grown at 30°C to an absorbance of 0.8–1.0 at 600 nm. Then, 1 mM of isopropyl-β-D-thiogalactopyranoside was added to induce the protein expression, and the bacteria were allowed to grow to the stationary phase. Protein concentrations were determined by the method of Bradford (17) on the cell lysate and on the partially purified hQR$_2$ (40–75% saturated ammonium sulfate).

Inhibition of hQR$_2$ and rQR$_1$ by Benzo(a)pyrene, Dicumarol, and Cibacron Blue. Inhibition of partially purified recombinant hQR$_2$ (specific activity 15 μmol per min/mg of protein) was compared with rQR$_1$ purified from livers of azo-dye-induced rats

with a specific activity 1875 μmol per min/mg of protein (22). The stock solutions of the inhibitors were prepared in ethanol. Appropriate concentrations of inhibitors were obtained by diluting stock solutions prepared in ethanol and adding 10 μl of these solutions to the reaction mixture containing 50 mM Tris·HCl buffer at pH 8.5, 30 μM menadione, and 80 μM *N*-methyldihydronicotinamide in a total volume of 1.0 ml. The reactions were carried out at 25°C and monitored at 360 nm for both QR$_1$ and QR$_2$, since *N*-alkyldihydronicotinamides are also good hydride donors for QR$_1$ (11).

RESULTS AND DISCUSSION

Purification of Bovine Kidney QR$_2$. The enzyme was purified by successive ammonium sulfate fractionation, heat treatment, and hydrophobic (phenyl-Sepharose) and ion-exchange chromatographies, as described in *Experimental Procedures*. These steps resulted in a 3100-fold increase in specific activity to give a final product that catalyzed the oxidation of 30 μmol of *N*-methyldihydronicotinamide per min/mg of protein with menadione as electron acceptor, which is comparable to the specific activity reported by Liao *et al.* (4) with *N*-propyldihydronicotinamide as substrate. The rates of reaction of bQR$_2$ with *N*-methyl- and *N*-propyldihydronicotinamide are similar (4). SDS/PAGE of the purified enzyme displayed a single band migrating at the position corresponding to ≈22 kDa (Fig. 1). We believe that this value underestimates the true molecular weight, since QR$_2$ is very hydrophobic, as indicated by its tight binding to phenyl-Sepharose (this work) and its exceedingly potent inhibition by polycyclic aromatic hydrocarbons and phenolic steroids (2, 4). It is well known that hydrophobic proteins tend to bind higher quantities of SDS and consequently migrate more rapidly than do most common proteins (23).

The homogeneity of the purified enzyme was further corroborated by the presence of a single sharp peak on reverse-phase HPLC in 0.1% TFA in water/acetonitrile (Fig. 1). The bright yellow solutions of the purified enzyme showed an absorption spectrum in the range of 350–550 nm, which is characteristic of flavoproteins, as reported earlier by Liao *et al.* (4), who identified the flavin as FAD. The flavin appears to be very tightly bound to the protein, since extensive chromatographic procedures (ion-exchange and hydrophobic chromatography in the presence of small amounts of ethanol, as well as gel filtration) failed to remove FAD from the active site, and the activity of the purified enzyme was not further increased by added FAD.

Amino Acid Sequence of Tryptic Peptides of bQR$_2$. The fractions containing the purified bQR$_2$ that had been subjected to reverse-phase HPLC in TFA/acetonitrile were evaporated
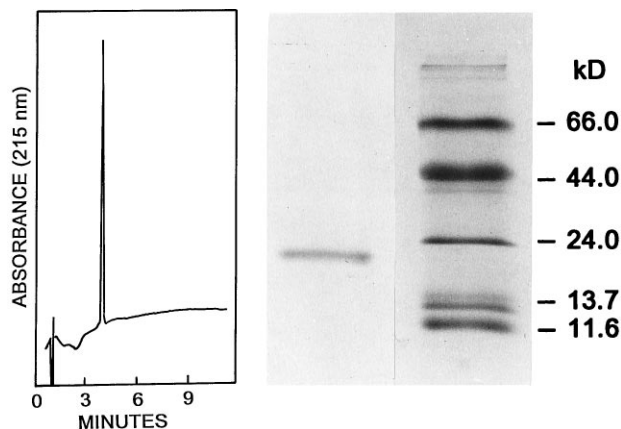

FIG. 1. Reverse-phase HPLC (*Left*) and SDS/PAGE (*Right*) of purified bovine kidney QR$_2$. The single band observed on the SDS/PAGE migrates at 22.0 kDa. The molecular weight standards are β-galactosidase (11.6 kDa), RNase (13.7 kDa), trypsinogen (24 kDa), ovalbumin (44 kDa), and BSA (66 kDa). The HPLC of purified bQR$_2$ shows a single absorption peak at 215 nm.
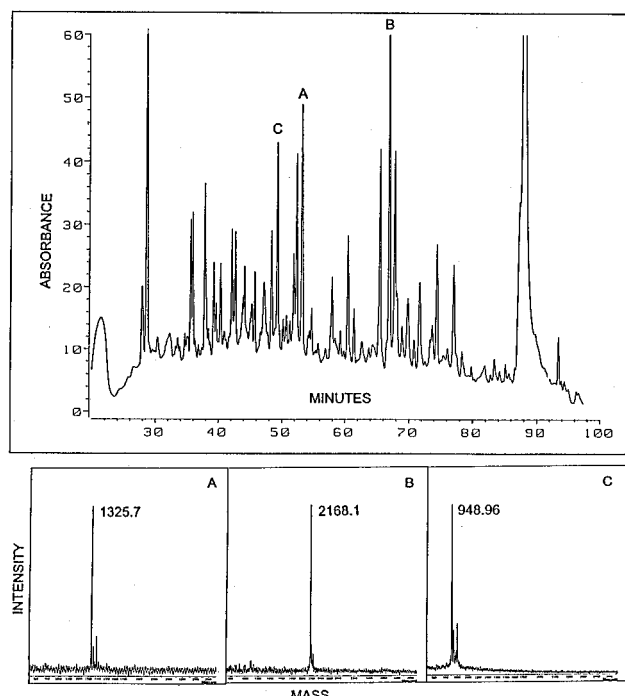
FIG. 2.   Tryptic mapping of homogeneous beef kidney QR_2. (*Upper*) HPLC trace from a Zorbax C_{18} column monitored at 215 nm is shown and three peptides selected for MALDI-MS and Edman sequencing are identified as A, B, and C (Table 1). Simultaneous monitoring at 277 nm and 292 nm (not shown) indicated the presence of tyrosine residues in A and B, and a tryptophan residue in C. (*Lower*) MALDI-MS (20) of peptide fragments A, B, and C. The molecular weights and Edman sequences of the peptides are given in Table 1.

to a small volume in a vacuum centrifuge. The material was then digested with trypsin and chromatographed on a Zorbax C_{18} column. UV absorption showed nearly 50 well-resolved peaks (Fig. 2). Three peaks (designated A, B, and C) were subjected to MALDI-MS (Fig. 2) and Edman sequencing (Table 1). There was excellent agreement between the calculated weights of the peptides (containing 11, 20, and 8 amino acids, respectively) and the corresponding MALDI-MS M/z (M + 1) determinations (Table 1). The three peptides derived from bQR_2 had a high degree of identity (75–91%) with the deduced sequence of hQR_2 cDNA, and a lesser yet highly significant homology to hQR_1 (5) (Table 1 and Fig. 3). On gel filtration, bQR_2 migrated at 40 kDa (not shown) indicating that this enzyme is a homodimer like QR_1.

Cloning, Sequencing, and Overexpression of hQR_2. The hQR_2 gene was amplified from total RNA of liver by reverse

Table 1.   Comparison of sequences of tryptic peptides of bQR_2 with hQR_1 and hQR_2

Peptide (position)		Amino acid		
		Sequence	Identity with bQR_2	Mol. wt. MALDI (calculated)
A	bQR_2	VLIVYAHQEPR	–	1325.7
(6–16)	hQR_2	∗∗∗∗∗∗∗∗∗∗K	10/11	(1324.5)
	(hQR_1	A∗∗∗L∗∗S∗RT)	6/11	
B	bQR_2	DITGTLSNPGFFNYGVEAHK		2168.1
(55–74)	hQR_2	∗∗∗∗∗∗∗∗∗EV∗∗∗∗∗∗∗T∗E	16/20	(2167.3)
	(hQR_1	∗∗∗∗K∗KD∗AN∗Q∗PA∗SVL)	9/20	
C	bQR_2	MVASWAQR		949.0
(204–211)	hQR_2	∗∗∗A∗S∗∗	6/8	(948.1)
	(hQR_1	ILEG∗KK∗)	2/8	

∗, amino acids identical with those of peptides of bQR_2. Mol. wt., molecular weight.

transcription–PCR with the use of two primers corresponding to the N-terminal and C-terminal sequences (see *Experimental Procedures* and ref. 5). The crude *E. coli* lysate of an overnight culture, induced with isopropyl-β-D-thiogalactopyranoside when it had reached an absorbance at 0.8–1.0 at 600 nm, had a specific activity of 1.6 μmol per min/mg of protein with *N*-methyldihydronicotinamide as hydride donor and menadione as acceptor. The enzyme was purified from the crude lysate as follows: nucleic acids were precipitated with 0.1% polyethyleneimine, and the supernatant fluid was then fractionated with solid ammonium sulfate. The 35–65% fraction, which contained most of the activity, was applied to a phenyl-Superose Fast Flow column and eluted with an increasing glycerol gradient. Pooled fractions were concentrated and applied to a Q Sepharose Fast Flow column, and eluted with a 0–200 mM NaCl gradient. Fractions with activity were combined and applied to a Mono Q column and eluted with a 0–175 mM NaCl gradient, and then subjected to gel filtration on a Superose 12 column. SDS/PAGE of the purified hQR_2 showed one major band with an apparent molecular weight of 24.6 kDa (calculated 25,951) and one minor higher molecular weight contaminant (Fig. 4). The specific activity of the purified enzyme was 242 μmol per min/mg of protein.

Properties of Recombinant hQR_2. In sharp contrast to the high sensitivity of QR_1 to inhibition by dicumarol (K_i = 1–2 nM; ref. 24) and Cibacron blue (K_i = 0.2–0.5 μM) (14, 22, 25), only marginal inhibition of QR_2 was observed with 10 μM dicumarol or Cibacron blue, whereas very potent inhibition ($\approx$75%) was observed with 0.1 μM benzo(*a*)pyrene (Fig. 5). The high affinity of hQR_2 for hydrophobic ligands is in accord with the truncation of the C-terminal portion of QR_2, which binds the pyrophosphate moiety of NAD(P)H in QR_1 (see below and Fig. 6). For both types of enzymes, the affinity for FAD is very high, because the FAD is not removed by a variety of chromatographic steps. The spectrum of purified hQR_2 showed absorbances characteristic of flavins in the 350–500 nm range. However, the peak absorbances of FAD are significantly red shifted when bound to hQR_2, i.e., from 450 to 456 nm and from 375 to 392 nm for the two major peaks. Thus, the truncation does not appear to affect FAD binding. The affinity for dicumarol appears to reside in major part in the C-terminal tail, since truncation of this tail in QR_2 significantly reduces the affinity for this inhibitor.

As reported for the mouse, rat, and bQR_2, recombinant hQR_2 is also completely inert toward NADPH, NADH, and NMNH. That NMNH is neither a substrate nor an inhibitor of hQR_2 suggests that the introduction of only one negatively charged phosphate group abolishes binding of dihydronicotinamide derivatives to QR_2.

Comparison of Structures and Function of QR_1 and QR_2. The similarities and differences of kinetic properties, hydride donor specificities, and inhibitor profiles of QR_1 and QR_2 are consistent with the amino acid sequences of the two enzymes and the x-ray crystal structure of QR_1 (12). The high degree of sequence homology between hQR_2 and hQR_1 legitimizes such comparisons. The crystal structure of rQR_1 (12) and proteolytic digestion experiments (26) have indicated that this enzyme consists of two domains: a major catalytic N-terminal domain (amino acids 1–220) and a much smaller C-terminal domain (residues 221–274). Truncation of QR_2 at residue 231 occurs in a loop near the juncture of the two domains between β-sheets 8 and 9 (figure 1 of ref. 12). The two-domain structure is also consistent with the observations (26) that trypsin, *Staphylococcus aureus* protease, and chymotrypsin cleaved rQR_1 in the same region and produced similar major truncations at K239–K240 (27.0 kDa), D229–L230 (25.9 kDa), and F228–D229 (25.7 kDa), respectively. Moreover, the proteolytic N-terminal fragments retained the dimeric form and the binding affinity for FAD (26), similar to QR_2. The proximity to the active site of the C-terminus of QR_2, which stabilizes the adenine ring of NAD(P)H in QR_1, could explain the

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  1    VLIVY AHQEPR (Peptide A)                                    DITGTL
       ***** *****                                                ******
hQR₂ MAGKKVLIVY AHQEPKSFNG SLKNVAVDEL SRQGCTVTVS DLYAMNFEPR ATDKDITGTL
     * *   *** ** * ***    *  *   *    *  *  * ******* *    ***** *
hQR₁ MVGRRALIVL AHSERTSFNY AMKEAAAAAL KKKGWEVVES DLYAMNFNPI ISRKDITGKL

 61
     SNPGFFNYGV EAHK (Peptide B)
     *** ***** * *
hQR₂ SNPEVFNYGV ETHEAYKQRS LASDITDEQK KVREADLVIF QFPLYWFSVP AILKGWMDRV
      *   *    *   ***    *  **  ***  *   ****** **** ** ** ****** **
hQR₁ KDPANFQYPA ESVLAYKEGH LSPDIVAEQK KLEAADLVIF QFPLQWFGVP AILKGWFERV

121
hQR₂ LCQGFAFDIP GFYDSGLLQG KLALLSVTTG GTAEMYTKTG VNGDSRYFLW PLQHGTLHFC
     **         ** *      * * ** ***  * **   * * **   ** * * * ****
hQR₁ FIGEFAYTYA AMYDKGPFRS KKAVLSITTG GSGSMYSLQG IHGDMNVILW PIQSGILHFC

181                              MVASWAQ R (Peptide C)       231
                                *** * * *
hQR₂ GFKVLAPQIS FAPEIASEEE RKGMVAAWSQ RLQTIWKEEP IPCTAHWHFG Q
     ** ** **   *          *     *    ** ** * *           *
hQR₁ GFQVLEPQLT YSIGHTPADA RIQILEGWKK RLENIWDETP LYFAPSSLFD LNFQAGFLMK

241                          274
hQR₁ KEVQDEEKNK KFGLSVGHHL GKSIPTDNQI KARK
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FIG. 3.   Sequence and location of the three tryptic peptides (A, B, and C) obtained from purified bQR₂ compared with the deduced protein sequences of hQR₂ (231 amino acids; M_r 25,952) and hQR₁ (274 amino acids; M_r 30,832). The tryptic peptides correspond to the QR sequence as follows: A, residues 6–16; B, residues 55–74; C, residues 204–211. Among the 39 amino acids of the tryptic peptides sequenced there is 82% (32 of 39) identity between hQR₂ and the purified bQR₂. Note that this similarity is much closer than 49% identity between hQR₁ and hQR₂ in the overlapping region (231 amino acids from the N terminus).

greater hydrophobicity of the active site, which favors binding of hydrophobic substrates and inhibitors.

In analogy to QR₁ (12, 27), we anticipate that QR₂ will also promote a ping-pong mechanism resulting in a two-electron reduction, because the basic enzymatic machinery appears to be the same for the two enzymes. Furthermore, (*i*) the inhibition of QR₁ by dicumarol is linearly competitive with respect to NADH, but not to quinones (25), consistent with the observation that QR₂ lacks affinity for dicumarol and is unable to use NADH or NADPH, which are competitors with dicumarol; (*ii*) the x-ray crystal structure of rat liver QR₂ showed that Cibacron blue, a potent and competitive inhibitor of QR₁ with respect to NAD(P)H (22), occupied the same site as the
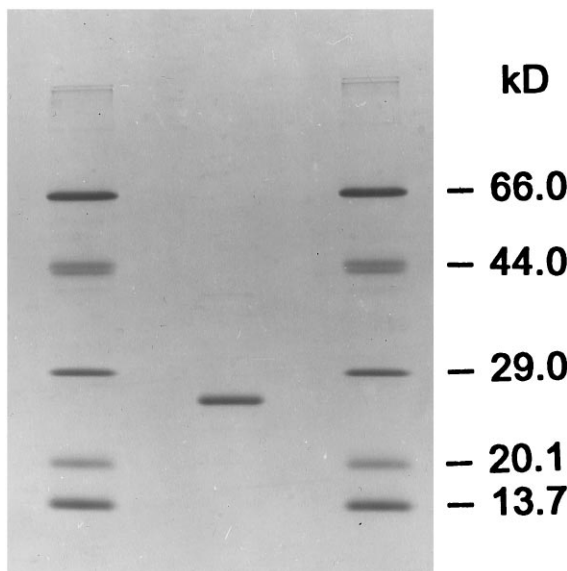

FIG. 4.   SDS/PAGE of purified hQR₂. The cloned, overexpressed, and purified hQR₂ migrated with a M_r 24.6 kDa. There is a trace contaminant of higher molecular weight. The standards are: RNase (13.7 kDa), soybean trypsin inhibitor (20.1 kDa), carbonic anhydrase (29 kDa), ovalbumin (44 kDa), and BSA (66 kDa).

nucleotides (12). This observation is also consistent with the ping-pong enzyme kinetics. Moreover, Cibacron blue is only a weak inhibitor of QR₂, and this enzyme does not bind to a Cibacron blue affinity column, in striking contrast to QR₁, which binds to Cibacron blue very tightly and was purified in a single step on this type of column (22); and (*iii*) the adenine ring of NADP⁺ and the anthraquinone ring of Cibacron blue interact with the C-terminal domain (residues 232 and higher), especially the main chain of the loop connecting strands 8 and 9 (L230–F236) (12). The binding site for the pyrophosphate moiety of NAD(P)H resides in this C-terminal tail. It is therefore of interest that the C-terminal 43 amino acids are exceedingly rich in the basic amino acid lysine (8 of 43 residues), whereas the remainder of the chain contains only 8 lysines among 231 amino acid residues. These observations suggest that QR₂ may be viewed as a C-terminal truncation analogue of QR₁, and that the absence of the carboxyl-terminal tail should favor the participation of smaller hydride donors as cofactors in the oxidoreduction reactions (Fig. 6).

There is substantial evidence that, by virtue of its obligatory two-electron reduction mechanism, QR₁ plays an important role in protecting cells against the toxicities of quinones and the reactive oxygen species that arise from oxidative cycling of quinones (8). QR₁ is highly induced in mammalian cells coordinately with other Phase 2 enzymes, resulting in protection against the toxic and neoplastic effects of electrophiles (7, 28). The role of QR₂ is much more obscure. It is only modestly inducible (5, 13) and its natural hydride donor is presumably *N*-ribosyldihydronicotinamide, which may arise from the hydrolytic breakdown of NADH or NADPH that occurs to varying degrees in a number of tissue extracts (3, 4). The quantitative importance of these reactions in intact tissues is not known. NMNH can be hydrolyzed by phosphatases to *N*-ribosyldihydronicotinamide. Significant quantities of oxidized *N*-ribosylnicotinamide can also arise from the enzymatic breakdown of NAD and NADP in mammalian tissues (3, 29),
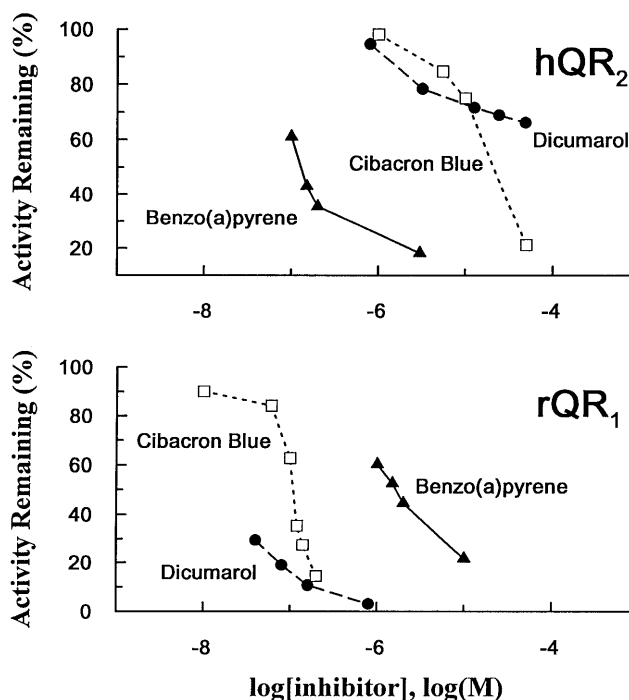
FIG. 5.   Striking differences between the inhibitor specificities of the two types of QR. (*Upper*) hQR₂. (*Lower*) rQR₁. Activity was measured with 30 μM menadione/80 μM *N*-methyldihydronicotinamide in 50 mM Tris·HCl buffer (pH 8.5) in the presence of 1% ethanol. Benzo(*a*)pyrene is a potent inhibitor of hQR₂, consistent with the results with rQR₂ and bQR₂ (1–4), whereas QR₁ is known to be very sensitive to inhibition by dicumarol and Cibacron blue (22, 24).

FIG. 6.   Three-dimensional structure of rQR$_1$ in complex with FAD and NADP$^+$ (12). The C-terminal 43 amino acids (232–274) are shown in red (*Left*), and have been truncated at the arrow (*Right*). Different colors of the backbone indicate 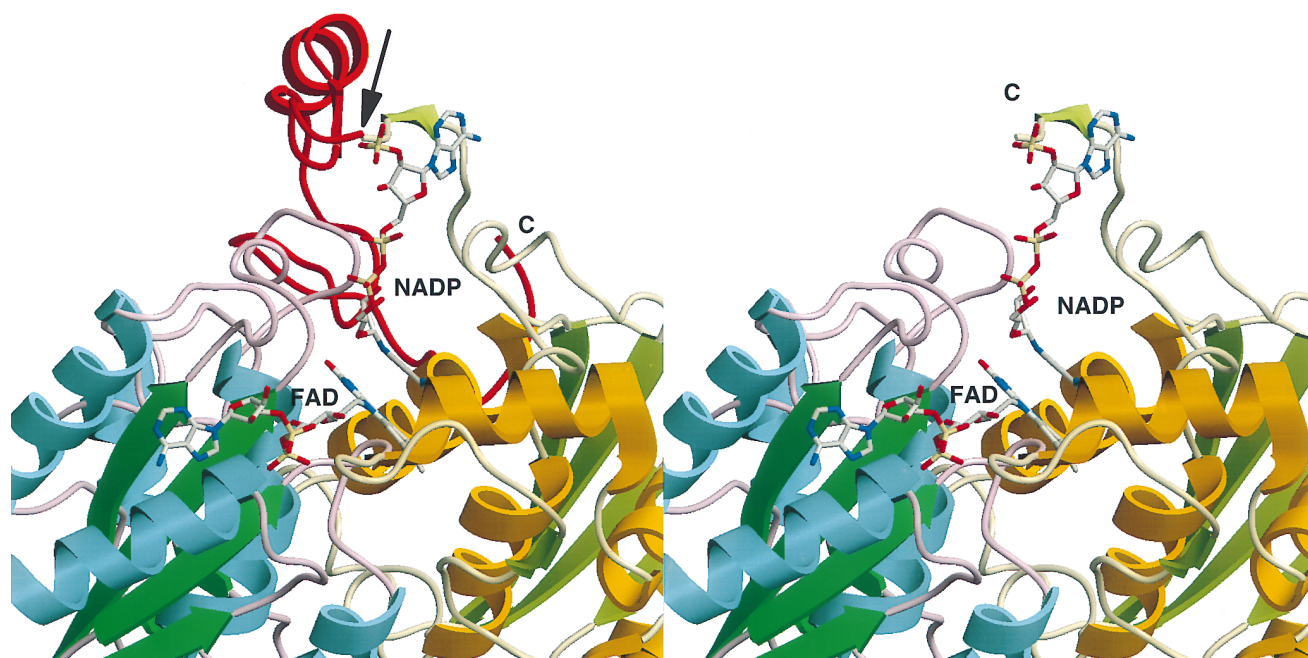the two different subunits. It can be seen that the truncated portion of QR$_2$ is critical for binding of the pyrophosphate moiety of the NAD(P)H cofactor. Note that the contact regions between the enzyme and FAD are presumably preserved in QR$_2$ (*Right*), consistent with the tight binding of FAD. The structural comparison between rQR$_1$ and hQR$_2$ is appropriate since the homology between rQR$_1$ and hQR$_1$ is very high (85% identity).

or by enzymatic synthesis from nicotinamide in the presence of phosphoribosylpyrophosphate (for review see ref. 30). To our knowledge, however, no enzymes promoting reduction of *N*-ribosylnicotinamide have been described in animal tissues, although Kaplan and colleagues (31) have described this reaction in *Pseudomonas fluorescens*. A search for such enzymes in animal tissues may therefore be rewarding.

Nicotinamide is released from NAD by a number of important cellular reactions, including nicotinamide nucleotide hydrolases, ADP ribosyltransferases transferases, the extensive ADP ribosylations of proteins, and reactions involved in the formation of poly(ADP) ribose (30). Both endogenous and exogenous nicotinamide are methylated to *N*-methylnicotinamide in tissues, and this is a normal excretory product found in the urine. Nevertheless, there is no evidence for the occurrence of *N*-alkylnicotinamide reducing enzymes in animal tissues. Although the physiological significance of QR$_2$ is unclear, the catalytic properties of QR$_1$ and QR$_2$ differ mostly in hydride donor specificity. Because QR$_1$ is believed to play an important role in the reductive bioactivation of quinone and nitro group-containing cytotoxic agents (10, 11, 13, 32), QR$_2$ may play a similar role provided the appropriate hydride donors are available.

In conclusion, an old flavoenzyme discovered more than 30 years ago, which was not even officially recognized by the *International Committee on Enzyme Nomenclature*, was identified as type 2 QR. Both types of QR appear to have the same catalytic machinery for FAD-mediated hydride transfer, whereas their substrate and inhibitor specificities are strikingly different as a result of truncation of the C-terminal 43 amino acid residues in QR$_2$ (corresponding to the C-terminal domain of QR$_1$).

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