Rat Liver NAD(P)H:Quinone Reductase Nucleotide Sequence Analysis of a Quinone Reductase cDNA Clone and Prediction of the Amino Acid Sequence of the Corresponding Protein*

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We have determined the nucleotide sequence of a cDNA clone, pDTD55, complementary to rat liver quinone reductase mRNA (Williams, J. B., Lu, A. Y. H., Cameron, R. G., and Pickett, C. B. (1986) J. Biol. Chem. 261, 5524-5528). The cDNA clone contains an open reading frame of 759 nucleotides encoding a polypeptide comprised of 253 amino acids with a Mr of 28,564. To verify the predicted amino acid sequence of quinone reductase, we have been able to align the amino acid sequences of a cyanogen bromide digest of the purified enzyme to the sequence deduced from the cDNA clone. A comparison of the quinone reductase sequence with other known flavoenzymes did not reveal a significant degree of amino acid sequence homology. These data suggest that the quinone reductase gene has evolved independently from genes encoding other flavoenzymes.

Quinone reductase, formerly called DT-diaphorase (NAD(P)H:quinone oxidoreductase, EC 1.6.99.2) catalyzes the two-electron reduction of quinones and quinonoid compounds to hydroquinones (1-4). This flavoprotein has an apparent molecular weight of 54,000 and consists of two apparently identical subunits, each containing one FAD (3). Studies utilizing isolated hepatocytes and subcellular fractions have demonstrated that the cytotoxic effects mediated by the one-electron reduction of menadione by NADPH-cytochrome P-450 reductase can be diminished by the addition of quinone reductase (5, 6).

Recently, our laboratory has constructed a cDNA clone complementary to quinone reductase mRNA (7). We have utilized the cDNA clone to demonstrate that the level of quinone reductase mRNA is elevated in rats treated with 3-methylcholanthrene and in persistent hepatocyte nodules induced by chemical carcinogens (7, 8). Southern blot analysis of genomic DNA suggests that the quinone reductase gene is hypomethylated in nodular tissue compared to normal liver tissue surrounding the nodules (7).

In the present study, we have determined the nucleotide sequence of the cDNA clone, pDTD55, and have predicted the amino acid sequence of rat liver cytosolic quinone reductase. The authenticity of the cDNA clone has been confirmed by comparing the predicted amino acid sequence with amino acids obtained from conventional protein sequence analysis of a cyanogen bromide digest of purified enzyme. Surprisingly, we have found no significant sequence homology between quinone reductase and other flavoenzymes.

MATERIALS AND METHODS

Nucleotide Sequence Analysis of pDTD55—Appropriate restriction fragments were 5' (NcoI, Ball and HindIII) or 3' (PstI) end-labeled and subjected to the chemical cleavage method of Maxam and Gilbert (9). Purified PstI fragments of pDTD55 were cloned into M13 phase and sequenced by the Sanger di-deoxy-sequencing method (10). An oligonucleotide, 17 nucleotides long, was synthesized on a Solid Support Synthesizer (Biosearch) and purified by electrophoresis on a 15% polyacrylamide gel. The 17-mer was used in place of the universal primer in certain sequencing runs.

Determination of Amino Acid Composition—The amino acid composition was determined on a Beckman amino acid analyzer (model 121MB) using standard methodology.

Amino Acid Sequence Analysis—Quinone reductase was subjected to cyanogen bromide digestion (11) and the mixed digest sequenced in the Applied Biosystems gas-phase sequenator (model 870A) according to the manufacturer's specifications. High performance liquid chromatography was used to quantitate the phenylthiobimidothioin derivatives produced at each step (12).

Computer Analysis—The sequence comparison program of Intelligenetics using the Align command (Needleman and Wunsch homology search algorithm (13)) and Search command (Queen-Korse algorithm (14)) were used in this study to compare the sequence of quinone reductase with other flavoenzymes.

RESULTS AND DISCUSSION

DNA Sequence Analysis of the Quinone Reductase cDNA Clone—The quinone reductase cDNA clone, pDTD55, was constructed from mRNA prepared by polyosomal immunosorption techniques (7). The length of the cDNA insert in the clone was 1900 bp1. The strategy for DNA sequence analysis is presented in Fig. 1 (Appendix). The solid arrows represent the sites used for 5' or 3' end labeling and sequence analysis by the Maxam-Gilbert chemical sequencing procedure (9), whereas the dashed lines represent regions of the cDNA insert sequenced by the di-deoxy method (10). Although the total length of the insert in pDTD55 was originally reported to be 1900 bp (7), we found that the sequence 5' to the NcoI site was a 400-bp duplication of the sequence from NcoI toward the 3' end of the clone. This duplicated sequence most likely arose during second strand synthesis when the hairpin loop generated during first strand synthesis was not made blunt end by DNA polymerase I. Therefore, the actual length of the cDNA insert in pDTD55 is 1400 bp.

Analysis of the nucleotide sequence revealed a single open reading frame (ORF) that encodes 253 amino acids with a Mr of 28,564.

1 The abbreviation used is: bp, base pairs.
reading frame of 759 bp encoding a protein comprised of 253 amino acids (Fig. 2, Appendix). Although there is an in-frame Met (ATG) codon 78 bp upstream from the Met codon assigned as the initiation codon, the 78-bp sequence contains two termination codons in the reading frame. Hence, the 78-bp sequence is not part of the open reading frame. In addition to the coding sequence, the cDNA clone contains 113 nucleotides of the 5'-untranslated region and 522 nucleotides of the 3'-untranslated region.

**Amino Acid Sequence Analysis of Cyanogen Bromide Fragments of Quinone Reductase**—Initial efforts to verify our predicted amino acid sequence by NH2-terminal sequence analysis of the purified enzyme proved unsuccessful. Numerous sequencing runs suggested that the NH2-terminal amino acid was blocked. Consequently, a cyanogen bromide digest was performed and the mixed digest sequenced utilizing the gas phase sequenator. The amino acid yields at each step from cycle 1 to 33 were quantitated and then aligned with the predicted amino acid sequence (Table I, underlined amino acids). At each step in the sequencing analysis, the amino acids generated from conventional protein sequencing could be aligned with the amino acid sequence predicted from DNA sequence analysis. The only exception is the third amino acid, Gln, in the second underlined fragment of the deduced amino acid sequence. Conventional amino acid sequencing indicated a Lys in that position. The amino acid composition of purified DT-diaphorase also agreed with the composition predicted from DNA sequence analysis of the cDNA clone (Table II).

Although there have been suggestions that multiple quinone reductases exist in rat liver (15), our cloning data suggest the presence of a single form (7). These findings are in contrast to the isozyme composition in mice. Prochaska and Talalay (16) have purified two forms of the enzyme from mouse liver cytosol, which are immunochemically similar. We cannot rule out, however, in the rat that multiple quinone reductases exist and are encoded by a gene or gene family that is unrelated to the gene encoding the quinone reductase characterized in this study. Finally, since all the amino acids from conventional protein sequencing could be accounted for in the deduced amino acid sequence, we also believe that each subunit of the enzyme is identical or extremely similar.

**Comparison of the Amino Acid Sequence of Quinone Reductase to Other Flavoenzymes**—Porter and Kasper (17, 18) have made extensive comparisons of the sequence of NADPH cytochrome P-450 reductase to other flavoenzymes. They have found that the tentative FAD-binding domain of the reductase, residues 267-678, shows a high degree of similarity to ferredoxin NADP+ reductase and NADH-cytochrome b5 reductase. We have compared the amino acid sequence of quinone reductase to other flavoenzymes, which include NADPH cytochrome P-450 reductase (17), NADH cytochrome b5 reductase (19), ferredoxin NADP+ reductase (20), glutathione reductase (21), p-hydroxybenzoate hydroxylase (22), Desulfovibrio vulgaris flavodoxin (23), Clostridium MP flavoprotein (24), pig kidney D-amino acid oxidase (25), and Escherichia coli fumarate reductase (26). Using the alignment algorithm of Needleman and Wunsch (13), we did not observe significant amino acid sequence homology between quinone reductase and the other flavoenzymes. These findings suggest that the quinone reductase gene evolved from an ancestral gene that was distinct from the ancestral gene(s) encoding other flavoenzymes. From a functional viewpoint, the quinone reductase is unique in that it accepts electrons from both NADH and NADPH with equal efficiency, whereas many other flavoproteins are highly specific to electron donors. In addition, dicumarol is a highly specific inhibitor of quinone reductase (5, 6), and it inhibits the enzyme by interfering with the electron transfer from NADPH to FAD (3). Thus, the

### Table I

**Amino acid sequence analysis of CNBR digest of quinone reductase**

| Cycle | Fragment A | Yield | Fragment B | Yield | Fragment C | Yield | Fragment D | Yield |
|-------|------------|-------|------------|-------|------------|-------|------------|-------|
|       | nmol       | nmol  | nmol       | nmol  | nmol       | nmol  | nmol       | nmol  |
| 1     | Asn (1.74) | Lys   | (1.31)     | Tyr   | (4.18)     | Tyr   |             |       |
| 2     | Val (2.20) | Glu   | (0.80)     | Asp   | (0.78)     | Ser   | (0.78)     |       |
| 3     | Ile (2.30) | Ala   | (1.25)     | Lys   | (1.45)     | Leu   | (1.84)     |       |
| 4     | Leu (2.60) | Ala   | (1.29)     | Gly   | (1.20)     | Gln   | (1.53)     |       |
| 5     | Gly (0.96) | Val   | (1.15)     | Pro   | (0.84)     | Gly   | (0.82)     |       |
| 6     | Pro (1.01) | Glu   | (0.42)     | Phe   | (1.48)     | Val   | (1.53)     |       |
| 7     | Ile (2.11) | Ala   | (1.55)     | Gln   | (1.67)     |       |           |       |
| 8     | Gln (1.43) | Leu   | (1.01)     | Asn   | (0.86)     | Gly   | (0.78)     |       |
| 9     | Ser (0.39) | Lys   | (1.62)     | Lys   |             | Asp   | (0.27)     |       |
| 10    | Gly (0.50) | Lys   | (1.92)     |       |             |       |           |       |
| 11    | Ile (0.94) | Lys   | (1.09)     | Thr   | (0.42)     |       |           |       |
| 12    | Leu (1.71) | Gly   | (0.38)     | Leu   | (1.54)     |       |           |       |
| 13    | Arg (0.26) |       |             |       |             |       |           |       |
| 14    | Phe (0.83) |       |             |       |             |       |           |       |
| 15    | Val (0.65) |       |             |       |             |       |           |       |
| 16    | Gly (0.34) |       |             |       |             |       |           |       |
| 17    | Phe (0.75) |       |             |       |             |       |           |       |
| 18    | Gln (0.62) |       |             |       |             |       |           |       |
| 19    | Val (0.66) |       |             |       |             |       |           |       |
| 20    | Leu (1.32) |       |             |       |             |       |           |       |
| 21    | Glu (0.91) |       |             |       |             |       |           |       |
| 22    | Pro (0.34) |       |             |       |             |       |           |       |
| 23    | Gln (0.36) |       |             |       |             |       |           |       |
| 24    | Leu (0.46) |       |             |       |             |       |           |       |
| 25    | Val (0.44) |       |             |       |             |       |           |       |
| 26    | Tyr (0.34) |       |             |       |             |       |           |       |
| 27    | Ser (0.09) |       |             |       |             |       |           |       |
| 28    | Ile (0.22) |       |             |       |             |       |           |       |
| 29    | Gly (0.13) |       |             |       |             |       |           |       |
| 30    | Ser (0.03) |       |             |       |             |       |           |       |
| 31    | Arg (0.06) |       |             |       |             |       |           |       |
| 32    | Pro (0.09) |       |             |       |             |       |           |       |
| 33    | Pro (0.10) |       |             |       |             |       |           |       |

*The amino acids in Fragments A–D have been aligned according to the deduced amino acid sequence of quinone reductase. The yield of each amino acid at each step is presented in parenthesis.*

### Table II

**Amino acid composition of quinone reductase**

| Amino acid | Deduced from | DNA sequence | Analysis* |
|------------|--------------|--------------|-----------|
| Lys        | 20           | 20.3         |           |
| His        | 4            | 4.4          |           |
| Arg        | 7            | 8.7          |           |
| Asp/Asn    | 20           | 18.5         |           |
| Thr        | 8            | 8.7          |           |
| Ser        | 17           | 16.9         |           |
| Glu/Gln    | 33           | 30.2         |           |
| Pro        | 14           | 12.9         |           |
| Gly        | 20           | 19.4         |           |
| Ala        | 17           | 19.7         |           |
| Cys        | 6            | ND*          |           |
| Val        | 18           | 16.2*        |           |
| Met        | 5            | 2.1          |           |
| Ile        | 12           | 16.1*        |           |
| Leu        | 26           | 26.4         |           |
| Phe        | 15           | 14.9         |           |
| Tyr        | 10           | 9.5          |           |
| Trp        | 6            | ND           |           |

*The purified DT-diaphorase sample was subjected to a 20-h hydrolysis.

ND, not determined.

Val-Ile peptide bands are incompletely hydrolyzed under the conditions used.

*Met and Cys residues are partially or completely destroyed under the hydrolysis conditions used.
FAD-binding domain of the quinone reductase must be considerably different from other flavoproteins in order to exhibit the unique electron accepting properties and its high affinity for dicumarol.

In summary, the quinone reductase cDNA clone characterized in this study will allow us to characterize the structural gene encoding the enzyme and to elucidate the regulatory elements that make the gene responsive to polycyclic aromatic hydrocarbons.

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APPENDIX

Fig. 1. DNA sequencing strategy of the quinone reductase cDNA clone. Appropriate restriction fragments of pDTD55 were 5' (NcoI, BglII, and HindIII) or 3' (PstI) end-labeled and subjected to nucleotide sequence analysis by the chemical cleavage method (9). The direction of DNA sequencing from each restriction endonuclease site is indicated by a solid line. Purified PstI fragments of pDTD55 were cloned into M13 phage and sequenced by the dideoxy-sequencing method (10). The broken line represents the direction of dideoxy sequencing. All fragments were sequenced two to three times.
FIG. 2. DNA sequence analysis of the quinone reductase clone and prediction of the amino acid sequence of the protein. The nucleotide sequence was determined as described under "Materials and Methods." The amino acid sequence was deduced by computer analysis of the DNA sequence. The underlined amino acids correspond to amino acid sequences within quinone reductase that correspond to the amino acids determined by protein sequence analysis of the cyanogen bromide digest of the purified protein (see Table I).