**Structural Studies and Isolation of cDNA Clones Providing the Complete Sequence of Rat Liver Dihydropteridine Reductase**

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The cleavage of reductively alkylated rat liver dihydropteridine reductase with cyanogen bromide afforded a mixture of peptides, six of which (CB-1 to CB-6) were isolated and purified by C8 reverse-phase high performance liquid chromatography. Portions of peptides CB-1, CB-4, and CB-6 were sequenced by automated Edman degradation and high performance liquid chromatography and the carboxyl-terminal region by conventional procedures. Further proteolytic digestion of CB-6 and isolation of the products afforded a seven-amino acid peptide. A low degeneracy probe comprising 20 nucleotides was synthesized from the sequence of this peptide and was used to screen a rat liver cDNA expression library constructed in the vector λgt10. Positive clones were isolated, and detailed examination of five of these by restriction endonucleases and dideoxy sequence analyses allowed identification of the entire coding region for dihydropteridine reductase. The gene was found to code for a protein of 240 amino acids (excluding the methionine initiator) of \( M_r = 25,420 \). Each of the sequences corresponding to the peptides CB-1, CB-4, CB-6, and the carboxyl terminus were identified in the deduced protein sequence. The rat enzyme is highly homologous to the human dihydropteridine reductase; the two proteins differ in only 10 amino acids, and all are conservative substitutions. In contrast, the sequence shows little homology with that of mammalian dihydrofolate reductase: reduced pyridine nucleotide-requiring enzymes with superficial mechanistic similarities.

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**Dihydropteridine reductase (EC 1.6.99.7)** promotes the NADH-mediated reduction of “quinoid” dihydrobipterin to tetrahydrobipterin in mammalian tissue. The tetrahydro-derivative is a required cofactor in the enzymatic hydroxylations of phenylalanine, tyrosine, and tryptophan, and the integrity of the coupled reductase-hydroxylase cycles is vital to the host as they provide essential steps in the biosyntheses of dopamine, epinephrine, and serotonin (1–4). Disturbances of this pathway can lead to either elevated catecholamine or cause phenylketonuria, a genetic defect in children which, if undetected, can lead to serious irreversible brain damage. This latter problem can result from an absence of a functional phenylalanine hydroxylase or dihydropteridine reductase and in some cases by deficiencies in pteridine cofactor biosynthesis (6, 7). Dihydropteridine reductase is of mechanistic interest as it requires an isomeric, structurally unstable quinonoid dihydropteridine as its substrate (8) in contrast to dihydrofolate, an NADPH-requiring enzyme, which acts upon the 7,8-dihydrofolate/pteridine isomer (9). Because of the clinical importance of dihydropteridine reductase and its superficial mechanistic similarities to dihydrofolate reductase, this laboratory has published several reports characterizing the enzyme isolated from rat liver (10, 11) and has presented preliminary x-ray crystallographic data (12). Recently, to assist in the purification of dihydropteridine reductase and to fully resolve three-dimensional characterization of the crystal structure, it was considered necessary to obtain the complete amino acid sequence. Cloning techniques were considered most propitious; therefore, this report describes the identification of a suitable peptide sequence from the purified enzyme from which a DNA probe was synthesized and then used to identify complementary sequences in a rat liver cDNA library constructed in λgt10. This approach ultimately allowed the isolation, characterization, and sequencing of the full dihydropteridine reductase coding region (720 base pairs), thus enabling deduction of the amino acid sequence of the enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials**—Reagents and supplies were from the following sources: Trizma (Tris base), Trizma-HCl, phenylmethylsulfonyl fluoride, TLCK; TPCK, NADH, phenyl-Sepharose, Sephadex G-100, Cibacron blue agarose, diithoerythritol, carboxypeptidase Y, pronase, pepsin, protease from *Staphylococcus aureus* strain V8 (Sigma); CNBr and EDTA (J. T. Baker Chemical Co.); iodoacetamide (Behring Diagnostics); DABITC (Fluka AG Chemische Fabrik); Dowex 50-H+ 1-X8 (Bio-Rad); rat livers (Pel-Freez Biologicals); [γ-32P]ATP (specific activity >7000 Ci/mol) (ICN Radiochemicals); [α-32P]dATP (specific activity >1000 Ci/mol) (Amersham Corp.); *Escherichia coli* Y1090, JM109, and DH5α, restriction endonucleases, E. coli DNA polymerase I (Klenow fragment), T4 DNA ligase, and T3, T4 polymerase I (New England Biolabs); M13mp18 and M13mp19**1** sequencing/ cloning kit (Pharmacia Biotechnology, Inc.). The rat liver cDNA library constructed in the vector λgt10 (13) was a generous gift of Dr. G. Howlett (University of Melbourne, Australia).

**Synthesis of the Nucleotide Probe**—This was kindly carried out by personnel at the Agouron Institute, La Jolla, CA, using an Applied Biosystems automatic synthesizer.

**Purification of Rat Liver Dihydropteridine Reductase**—The enzyme was purified by a refined technique based on a previously published

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank**2**/EMBL Data Bank with accession number(s) J0348L.

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1 The abbreviations used are: TLCK, sodium p-tosyl-L-lysine chloromethyl ketone; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone; SDS, sodium dodecyl sulfate; DABITC, 4-(dimethylamino)azobenzene-4'-isothiocyanate; HPLC, high performance liquid chromatography.
pyridine acetate, pH 5.6, containing 1% SDS and 0.1 mM norleucine. After centrifugation, the mixture was dialyzed against buffer prior to application to the second column where elution was achieved with 100 μM NADH in buffer. Appropriate fractions from the HPLC were pooled, lyophilized, and then applied consecutively to two Bio-Rad blue agarose columns (3.5 x 20 cm). Recovery from the first column was achieved with 2 M NaCl and combined active fractions dialyzed against buffer prior to application to the second column where elution was achieved with 100 μM NADH in buffer. Appropriate fractions from the HPLC were pooled, lyophilized, and then applied consecutively to two Bio-Rad blue agarose columns (3.5 x 20 cm). Recovery from the first column was achieved with 2 M NaCl and combined active fractions dialyzed against buffer prior to application to the second column where elution was achieved with 100 μM NADH in buffer. Appropriate fractions from the HPLC were pooled, lyophilized, and then applied consecutively to two Bio-Rad blue agarose columns (3.5 x 20 cm). Recovery from the first column was achieved with 2 M NaCl and combined active fractions dialyzed against buffer prior to application to the second column where elution was achieved with 100 μM NADH in buffer. Appropriate fractions from the HPLC were pooled, lyophilized, and then applied consecutively to two Bio-Rad blue agarose columns (3.5 x 20 cm). Recovery from the first column was achieved with 2 M NaCl and combined active fractions dialyzed against buffer prior to application to the second column where elution was achieved with 100 μM NADH in buffer. Appropriate fractions from the HPLC were pooled, lyophilized, and then applied consecutively to two Bio-Rad blue agarose columns (3.5 x 20 cm). Recovery from the first column was achieved with 2 M NaCl and combined active fractions dialyzed against buffer prior to application to the second column where elution was achieved with 100 μM NADH in buffer. Appropriate fractions from the HPLC were pooled, lyophilized, and then applied consecutively to two Bio-Rad blue agarose columns (3.5 x 20 cm). Recovery from the first column was achieved with 2 M NaCl and combined active fractions dialyzed against buffer prior to application to the second column where elution was achieved with 100 μM NADH in buffer. Appropriate fractions from the HPLC were pooled, lyophilized, and then applied consecutively to two Bio-Rad blue agarose columns (3.5 x 20 cm). Recovery from the first column was achieved with 2 M NaCl and combined active fractions dialyzed against buffer prior to application to the second column where elution was achieved with 100 μM NADH in buffer. Application of porcine pancreatic trypsinogen (2.2 x 12 cm) pre-equilibrated with 1 M (NH₄)₂SO₄ in buffer and elution with buffer alone afforded a product which, after concentration, was passed through Sephadex G-150 (2 x 120 cm) employing the same buffer. The standard buffer solution used throughout was 50 mM potassium phosphate, pH 6.8, containing 0.1% mercaptoethanol. The average yield of enzyme, homogeneous by SDS-polyacrylamide gel electrophoresis, was 25 mg of specific activity >300 units/mg.

Reductive Alkylation and Cyanogen Bromide Cleavage—According to the procedure of Crestfield et al. (15), enzyme (10 mg in 2 ml of standard buffer) was dialyzed against 0.1 M Tris-HCl containing 0.5 mM EDTA and then treated with urea to a final concentration of 6 M. A 16-fold molar excess of β-mercaptoethanol (relative to half-cystine content) was added, and the stirred solution was incubated under argon, in the dark, at 25°C for 2 h. A 10-fold molar excess of iodoacetamide was added, and the mixture was incubated for an additional 2 h to form a 100-fold excess of β-mercaptoethanol, dialysis against water overnight, and then lyophilized. Cleavage was carried out by the method of Prabh and Porter (16) by dissolving the sample in 70% formic acid (2 ml) containing a 100-fold molar excess of cyanogen bromide (relative to methionine content), and the stirred solution was incubated at 37°C for 16 h. The sample was then diluted 10-fold with water and lyophilized.

Amino Acid Analyses—Purified enzyme or peptide fragments were hydrolyzed with constant boiling HCl in vacuo at 110°C for 20–60 h. The amino acid compositions of the hydrolysates were analyzed with automatic amino acid analyzers, Beckman Models 119C and 6300.

SDS-Polyacrylamide Gel Electrophoresis—This procedure was carried out according to the method of Weber and Osborn (17) with the modification of using 12.5% gels containing 1.25% bisacrylamide and 6 M urea, which were run at a constant current of 3 mA/gel, fixed in 10% trichloroacetic acid, and stained with Coomassie R-250.

Enzyme Degradation and Peptide Isolation—Purified dihydropteridine reductase was reductively alkylated in the presence of 6 M urea and then cleaved with CNBr in 70% formic acid. SDS-polyacrylamide gel electrophoresis showed the presence of eight major bands ranging in M, from 2,500 to 20,000 (27). Quantitative HPLC analysis of the products on a C₈ reverse-phase column afforded the profile shown in Fig. 1 and allowed the isolation of six peptides (CB-1 to CB-6). Each of the peptides demonstrated a free amino-terminal residue indicating that the amino-terminal peptide was not sequenced via automated Edman degradation. The sequences, each of which is shown in Table 1, contained 38-39 amino acids of limited codon degeneracy and suggested that a suitable nucleotide probe could be designed.

Probe Synthesis—The peptide designated CB-6 gave the following sequence from the amino terminus: Pro-Glu-Ala-Asp-Phe-Ser-Ser-Thr-Pro-Leu-Glu-Phe-Leu-Val-Glu-Thr-Phe-His-Asp-Trp-Ile-Lys-Gly-Asn-Lys-Gly-Pro. Because some uncertainty often occurs with extended sequencing, further cleavage of this peptide was carried out using a protease from S. aureus strain V8 (28), and the product was subjected to HPLC on the C₈ reverse-phase column. Elution with an aqueous 2:1 acetonitrile/1-propanol linear gradient afforded five principal peaks (Fig. 2). Amino acid analysis showed that the second peak contained desaminomethyl amino acids of limited codon degeneracy and suggested that...
this region would be suitable for synthetic probe purposes. Its sequence was determined as Thr-Phe-His-Asp-Trp-Ile, with cleavage having occurred as expected adjacent to glutamic acid, and was identical to that contained in CB-6 above. As a prelude to cloning and sequencing the entire enzyme, a nucleotide probe 20 bases in length was therefore synthesized complementary to the expected messenger RNA sequence as follows.

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Th-Gl-His-Gl-Ile
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The oligonucleotide mixture was purified and phosphorylated with 32P as described under “Experimental Procedures.”

**Isolation and Sequence Analysis of Dihydropteridine Reductase Clones**—A λgt10 library of cDNA from rat liver messenger RNA was screened by hybridization using the labeled probe. Hybridization was observed to 38 plaques of approximately 250,000 screened. These were purified until 100% of the plaques hybridized with the probe. One of the isolates, λX DHPR38, was physically mapped by restriction enzyme analysis. The entire EcoRI fragment in this phage was inserted into M13 and sequenced using the oligonucleotide probe as a primer. Translation of the sequence corresponded exactly to that predicted from the sequence of the peptide confirming the identity of the isolate. The entire sequence of the dihydropteridine reductase structural gene was compiled from five separate bacteriophage isolates having different length cDNA inserts (Fig. 3).

**Amino Acid Sequence of Rat Dihydropteridine Reductase**—Translation of the open reading frame of the nucleotide sequence has provided the first complete amino acid sequence of rat dihydropteridine reductase (Fig. 4). The predicted molecular weight of the enzyme subunit (without the initiator methionine) is 25,420 (240 amino acids), which is very close to the literature reports for the rat enzyme of 25,500 (14) and almost identical to the human enzyme (M, = 25,760 (30)). The predicted sequence contains each of the regions determined by conventional protein sequencing as indicated in Fig. 4. The figure also illustrates the region complementary to the synthetic probe. Table I shows the predicted amino acid composition of rat liver dihydropteridine reductase, which agrees well with the previously published values for the enzyme. The amino acid composition of the human enzyme is also very similar, reflecting a high degree of sequence homology between the enzymes from the two sources.

| Nucleotide 5'- | A | T | C | A | G | T | C |
|---------------|---|---|---|---|---|---|---|
| Amino acid    | I | W | D |

**DISCUSSION**

This paper reports the isolation and sequencing of cDNA clones for rat dihydropteridine reductase and includes the determination of the complete coding sequence for the holoenzyme. CNBr cleavage of the reductively alkylated reduc-

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**Fig. 2.** C4 reverse-phase HPLC of the proteinase-cleaved CB-6 peptide. The peptide (~10 μmol) was incubated with 50 μg of *S. aureus* V8 protease in 50 μM ammonium bicarbonate, 2 mM EDTA for 3 h at 37°C, pH 8, and then chromatographed as described in the legend to Fig. 1. Peak B contained the peptide from which the nucleotide probe was derived.

**Fig. 3.** Sequencing strategy and partial restriction enzyme map of the dihydropteridine reductase cDNA derived from the nucleotide sequence in Fig. 4. The coding region is shown as a solid box. bp, base pairs.
ment, since no peptide with a blocked amino group was ever identified. In fact, only six peptides were isolated and identified. Peptide CB-6 was degraded further with S. aureus strain V8 protease and a peptide isolated by HPLC whose composition, reflecting low degeneracy of the genetic code, allowed a 20-base nucleotide probe to be synthesized preparatory to screening the Xgt10 rat liver cDNA library.

The sequence of dihydropteridine reductase deduced from the nucleotide sequence of the cloned gene is very similar to that of the human dihydropteridine reductase whose sequence has been recently reported from two laboratories (30, 31). Rat dihydropteridine reductase differs in 10 residues from one of the human enzyme sequences (30). The positions of substitution are randomly distributed throughout the protein, and large regions are completely conserved, e.g. residues 4–35, 75–168, and 170–214. The most significant difference between the amino acid sequences of the rat and human enzymes lies at the amino-terminal position, there being an additional three alanines and an alanine-serine replacement in the human protein, i.e. Ala-Ala-Ala-Ala-Ala-Ala (human) versus Ala-Ala-Ser (rat). Preliminary structural evidence (27) has suggested a pyroglobulysine-blocked terminal, but the current results support the concept of an acetylalanine block as has been suggested by other workers (29). Interestingly, none of the residues that differ are located in the conserved regions identified between dihydropteridine reductase and dihydrofolate reductase (30). The two human sequences reported contain a serine/threonine polymorphism at residue 50 (excluding the methionine initiator); this residue is serine (amino acid 47) in the rat enzyme.

In addition to possible site-directed mutagenesis experiments, the cloning and sequencing of the gene will allow complete resolution of the x-ray crystallographic structure (12). Specific information on the active sites might further be deduced from the related dihydrofolate reductase, although preliminary analysis has suggested little structural similarity to this latter enzyme despite its superficially similar mechanism of action (32). However, a region of homology between human dihydropteridine reductase and dihydrofolate reductase (residues 100–108 and 16–25, respectively), noted by Dahl et al. (30) as being receptive to the binding of a nicotinamide moiety, is identical in the rat enzyme (residues 96–105) and thus also suggests the presence of a preferred nucleotide-binding site. It is of interest to note the remarkable conservation of sequence.

![Image](image-url)

**Fig. 4.** Nucleotide and predicted amino acid sequence of dihydropteridine cDNA. The nucleotide sequence was determined using the dideoxy sequencing procedure of Sanger et al. (25). The deduced amino acid sequence numbers start from the amino-terminal alanine. Amino acids that differ from the nucleotide sequence are underlined, and the probe region is boxed.
Rat Liver Dihydropteridine Reductase Sequence

quence between rat and human reductases, suggesting a crucial integrity of structure pertinent to function. This also reinforces the probability that dihydropteridine reductase is an enzyme whose activity is essential to host survival.

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