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CLONING AND SEQUENCING OF cDNA ENCODING HUMAN SEPIAPTERIN REDUCTASE

–An Enzyme Involved in Tetrahydrobiopterin Biosynthesis–

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A full-length cDNA clone for sepiapterin reductase, an enzyme involved in tetrahydrobiopterin biosynthesis, was isolated from a human liver cDNA library by plaque hybridization. The nucleotide sequence of hSPR 8-25, which contained an entire coding region of the enzyme, was determined. The clone encoded a protein of 261 amino acids with a calculated molecular mass of 28,047 daltons. The predicted amino acid sequence of human sepiapterin reductase showed a 74% identity with the rat enzyme. We further found a striking homology between human SPR and carbonyl reductase, estradiol 17ß-dehydrogenase, and 3ß-hydroxy-5-ene steroid dehydrogenase, especially in their N-terminal region.

(6*R*)-(L-*erythro*-1',2'-dihydroxypropyl)-2-amino-4-hydroxy-5,6,7,8-tetrahydropteridine (tetrahydrobiopterin, BH_4) is an essential cofactor for aromatic amino acid monooxygenases, phenylalanine, tyrosine, and tryptophan hydroxylases (1-3). Recently BH_4 was also shown to be a cofactor in the generation of nitric oxides (4,5). Animals can synthesize BH_4 *in vivo* from GTP through several enzymatic reactions. As tyrosine and tryptophan hydroxylases are the rate limiting enzymes in the biosynthesis of monoamine neurotransmitters, their activities are regulated by several factors. The concentration of BH_4 within the nerve terminal is thought to be one of their regulatory factors (6). Abnormality in the metabolism of BH_4 results in certain neurological diseases such as atypical phenylketonuria (7), Parkinson's disease (8), and Alzheimer's disease (9).

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Sepiapterin reductase (7,8-dihydrobiopterin: $NADP^+$ oxidoreductase, EC 1.1.1.153; SPR) catalyzes the NADPH-dependent reduction of various carbonyl substances including derivatives of pteridines and belongs to a group of enzymes called aldo-keto reductases (10-12). Many reports support that SPR plays an important role for the biosynthesis of BH_4 by studies including pharmacological studies using a specific inhibitor and immunochemical experiments using a specific antibody (13-16). It is uncertain, however, whether two carbonyl groups on the side chain of 6-pyruvoyl tetrahydropterin, an intermediate involved in BH_4 formation, are

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                                                  -20       -10       -1
                                                  GCCGCCGGCGGAGAACAGGAGC

1         10        20        30        40        50        60
ATGGAGGGCGGGCTGGGGCGTGCTGTGTGCTTGCTGACCGGGGCCTCCCGCGGCTTCGGC
M  E  G  G  L  G  R  A  V  C  L  L  T  G  A  S  R  G  F  G

          70        80        90       100       110       120
CGGACGCTGGCCCCGCTCCTGGCCTCGCTGCTGTCGCCCGGCTCCGTGCTTGTCCTTAGC
R  T  L  A  P  L  L  A  S  L  L  S  P  G  S  V  L  V  L  S

         130       140       150       160       170       180
GCCCGCAACGACGAGGCACTGCGCCAGCTGGAGGCCGAGCTGGGCGCCGAGCGGTCTGGC
A  R  N  D  E  A  L  R  Q  L  E  A  E  L  G  A  E  R  S  G

         190       200       210       220       230       240
CTGCGCGTGGTGCGGGTGCCCCGCCGACCTGGGCGCCGAGGCCGGCTTGCAGCAGCTGCTC
L  R  V  V  R  V  P  A  D  L  G  A  E  A  G  L  Q  Q  L  L

         250       260       270       280       290       300
GGCGCCCTGCGCGAGCTCCCCCGGCCCAAGGGGCTGCAGCGACTGCTGCTTATCAACAAC
G  A  L  R  E  L  P  R  P  K  G  L  Q  R  L  L  L  I  N  N

         310       320       330       340       350       360
GCGGGCTCTCTTGGGGATGTGTCCAAAGGCTTCGTGGACCTGAGTGACTCCACTCAAGTG
A  G  S  L  G  D  V  S  K  G  F  V  D  L  S  D  S  T  Q  V

         370       380       390       400       410       420
AACAACTACTGGGCACTGAACTTGACCTCCATGCTCTGCCTGACTTCCAGCGTCCTGAAG
N  N  Y  W  A  L  N  L  T  S  M  L  C  L  T  S  S  V  L  K

         430       440       450       460       470       480
GCCTTCCCGGACAGTCCTGGCCTCAACAGAACCGTGGTTAACATCTCGTCCCTCTGTGCC
A  F  P  D  S  P  G  L  N  R  T  V  V  N  I  S  S  L  C  A

         490       500       510       520       530       540
CTGCAACCTTTCAAAGGCTGGGCGCTGTACTGTGCAGGAAAGGCTGCTCGTGATATGCTG
L  Q  P  F  K  G  W  A  L  Y  C  A  G  K  A  A  R  D  M  L

         550       560       570       580       590       600
TTCCAGGTCCTGGCGCTGGAGGAACCTAATGTGAGGGTGCTGAACTATGCCCCAGGTCCT
F  Q  V  L  A  L  E  E  P  N  V  R  V  L  N  Y  A  P  G  P

         610       620       630       640       650       660
CTGGACACAGACATGCAGCAGTTGGCCCGGGAGACCTCCGTGGACCCAGACATGCGAAAA
L  D  T  D  M  Q  Q  L  A  R  E  T  S  V  D  P  D  M  R  K

         670       680       690       700       710       720
GGGCTGCAGGAGCTGAAGGCAAAGGGGAAGCTGGTGGATTGCAAGGTGTCAGCCCAGAAA
G  L  Q  E  L  K  A  K  G  K  L  V  D  C  K  V  S  A  Q  K

         730       740       750       760       770       780
CTGCTGAGCTTACTGGAAAAGGACGAGTTCAAGTCTGGAGCCCACGTGGACTTCTATGAC
L  L  S  L  L  E  K  D  E  F  K  S  G  A  H  V  D  F  Y  D

         790       800       810
AAATAAGCCCATGTTTTTGGCTTCCTGAACC
K  *
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Fig. 1. The nucleotide sequence of cDNA encoding human sepiapterin reductase and the deduced amino acid sequence. The predicted amino acid sequence is shown below the nucleotide sequence by one-letter code.

reduced, *in vivo*, by the sequential action of SPR alone or by the two enzymatic reactions with SPR and aldose reductase (EC 1.1.1.21) (15,17). Molecular probes are required to study the metabolism of BH_4 from a new aspects.

Citron *et al.* reported a partial nucleotide sequence of rat SPR, which lacks the 5' sequence corresponding to the first 3 amino acids in the N-terminus of the protein (18). We determined 96% of the amino acid sequence of rat SPR including the N-terminal structure by protein analysis (19). The complete amino acid sequence of the mature form of rat SPR was provided by these two reports. In this paper, we report the cloning of a cDNA for SPR from human liver and the complete amino acid sequence of human SPR deduced from the nucleotide sequence of the cDNA. We also discuss the amino acid sequence homology among human SPR, human carbonyl reductase, and some dehydrogenases (Fig. 1).

MATERIALS AND METHODS

Polymerase Chain Reaction (PCR). First we cloned a cDNA for rat SPR by the reverse transcription-polymerase chain reaction (RT-PCR) method (20). Oligonucleotides for PCR were synthesized with an Applied Biosystems 381A DNA synthesizer. The sequence of primer A was 5'-GGGAATTCCATGGAGG G(ATGC)GGCAGGCTAGG-3' which was designed based on the N-terminal amino acid sequence of rat SPR previously described (19) and the nucleotide sequence reported by Citron *et al.* (18) with an additional *Eco*RI site. The sequence of primer B is 5'-GAGGATCCTTAAATGTCATAGAAGTCCACGTG-3' which is corresponding to the nucleotide sequence of 757-781 (18) including an additional *Bam*HI site. The total RNA (10 μg) from rat brain was reverse transcribed into the cDNA by Murine Moloney Leukemia Virus reverse transcriptase (Bethesda Research Laboratories) using primer B. The transcribed cDNA was amplified by the PCR using the primers A and B. We carried out 25 cycles of amplification and the profile of each cycle was: denaturation at 93°C for 3 min, then annealing at 60°C for 1.5 min and extension at 72°C for 3 min. The amplified DNA fragments were analyzed by agarose gel electrophoresis and cloned into a Bluescript KS (Stratagene) vector.

Screening of cDNA Library. A human liver cDNA library in λgt11 using a random primer and EcoRI linkers was a generous gift from Dr. Y. Ebina (The University of Tokushima, Japan). A total of 3×10^5 λ phage plaques on *E.coli* strain Y1088 lawn cells were screened. The phage plaques were lifted twice onto nylon membranes (Hybond-N, Amersham) and processed for hybridization. The membranes were prehybridized for at least 2 hrs at 65°C in 6 x SSC containing 5 x Denhardt's solution, 0.5% SDS and 0.1 mg/ml of salmon sperm DNA. Hybridization followed at 65°C for 20 hrs in the above specified buffer with a labeled probe. The membranes were washed with 2 x SSC containing 0.1% SDS at room temperature for 10 min twice, then soaked in the same solution at 42°C for 20 min twice. The cDNA clones with positive signal were purified and subcloned into EcoRI site of Bluescript KS (M13+).

DNA Sequence Analysis. The nucleotide sequences of the cDNA were determined by the dideoxynucleotide chain-termination method (21) using

Sequenase (United States Biochemical Corp., Cleveland, OH) and synthetic primers.

RESULTS AND DISCUSSION

First we amplified the cDNA for rat SPR by the PCR. Synthetic primers for the amplification were designed based on the nucleotide sequence of rat SPR (18) and the amino acid sequence of the mature form of rat SPR (19). Primer A was constructed based on the amino acid sequence of the first seven amino acids at the N-terminus with an additional *Eco*RI site. Primer B was corresponding to the seven amino acids at the C-terminus with an additional *Bam*HI site. Total RNA from rat brain was reverse transcribed using primer B. The single stranded cDNA generated was amplified by the PCR with primers A and B, and cloned into a plasmid vector.

The rat cDNA was labeled and then used for screening through plaque hybridization of 3 x 10^5 recombinants from a human liver cDNA library. Seven strongly hybridized clones were isolated, subcloned into the *Eco*RI site of a Bluescript vector and sequenced.

By analogy with the rat sequence, it was suggested that clone hSPR 8-25 contained an entire coding region of human SPR. Its sequence in both orientation was determined using synthetic primers. It contained an open reading frame encoding 261 amino acid residues following the first ATG, 22bp of 5' untranslated and 25 bp of 3' untranslated regions. A poly(A)$^+$ tail was not found in the clone, because the cDNA library used was made using random hexamers for priming cDNA synthesis, not but oligo(dT). In the previous paper, we determined the N-terminal sequence of the mature form of rat SPR, N-acetyl-Met-Glu-Gly-Gly (19). The amino acid sequence deduced from the nucleotide sequence of hSPR 8-25 began with Met-Glu-Gly-Gly-, suggesting that the ATG codon acts as the translation initiation signal. Based on the predicted amino acid sequence, the molecular mass was calculated to be 28,047 daltons for the protein.

The predicted amino acid sequence of human SPR was compared to that of the rat enzyme(18,19). A single gap was introduced into the human sequence for an optimal alignment. Human SPR revealed a 74% identity with the rat sequence (Fig. 2). A putative pterin binding site (Ala-Gly-Leu-Leu-Ser) suggested by Citron *et al.* (18) in rat SPR is underlined. As the amino acid sequence in human SPR corresponding to this sequence was Ala-Ser-Leu-Leu-Ser, the consensus sequence for a pterin-binding peptide should be Ala-Xxx-Leu-Leu-Ser. A NADPH binding consensus sequence (22), TL-L-G-G--G--L--L------L-L-D, spanning 27 amino acids from residues 14-40 was well conserved between human and rat SPR.

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        1       10        20        30        40        50        60
human   MEGG-LGRAVCLLTGASRGFGRTLAPLLASLLSPGSVLVLSARNDEALRQLEAELGAERS
        :::: :: ::: :::::::::: ::: :: :::::::: :::: :  ::::  ::
rat     MEGGRLGCAVCVLTGASRGFGRALAPQLAGLLSPGSVLLLSARSDSMLRQLKEELCTQQP

       61       70        80        90       100       110       120
human   GLRVVRVPADLGAEAGLQQLLGALRELPRPKGLQRLLLINNAGSLGDVSKGFVDLSDSTQ
        :: ::   :::: : : :::: : ::::::  :::::::::: :::::::    :
rat     GLQVVLAAADLGTESGVQQLLSAVRELPRPERLQRLLLINNAGTLGDVSKGFLNINDLAE

      121       130       140       150       160       170       180
human   VNNYWALNLTSMLCLTSSVLKAFPDSPGLNRTVVNISSLCALQPFKGWALYCAGKAARDM
        ::::::::::::::::   : ::  ::::  :::::::::::::::: ::::::::::
rat     VNNYWALNLTSMLCLTTGTLNAFSNSPGLSKTVVNISSLCALQPFKGWGLYCAGKAARDM

      181       190       200       210       220       230       240
human   LFQVLALEEPNVRVLNYAPGPLDTDMQQLARETSVDPDMRKGLQELKAKGKLVDCKVSAQ
        : :::: ::: :::: :::::::: ::::::::: ::  :  :: :   : ::::  :::
rat     LYQVLAVEEPSVRVLSYAPGPLDTNMQQLARETSMDPELRSRLQKLNSEGELVDCGTSAQ

      241       250       260
human   KLLSLLEKDEFKSGAHVDFYDK
        ::::::  : : :::::::::
rat     KLLSLLQRDTFQSGAHVDFYDI
```

Fig. 2. Comparison of the amino acid sequences of human and rat sepiapterin reductase. Identical residues are indicated by vertical dotted lines. Alignment was made by introducing a single gap at the residue 5 of the human sequence. A putative pterin binding site (16) is underlined.

Aldose reductase catalyzes the reduction of 2'-carbonyl group on the side chain of 6-pyruvoyl tetrahydropterin, while SPR does the reduction of both 1'- and 2'-carbonyl group of the same molecule. No obvious sequence homology, however, was found between human aldose reductase (23) and human SPR.

It is known that SPR has a dehydrogenase activity, a reverse reaction against reductase (10), and shows the activity of carbonyl reductases (12). Thus we compared the amino acid sequence of human SPR with human carbonyl reductase (EC 1.1.1.184) (24), human estradiol 17ß-dehydrogenase (EC 1.1.1.62) (25), and human 3ß-hydroxy-5-ene steroid dehydrogenase (EC 1.1.1.145) (26). They exhibits a striking homology with SPR especially in their N-terminal regions (Fig. 3). Gaps were introduced to obtain a maximal

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          1       10        20        30        40        50        60
SPR     1 MEGGLGRAVCLLTGASRGFGRTLAPLLASLLSPGSVLVL..SARNDEALRQLEAE.LGAE
EDH     1       A-T-V-I--C-S-I-LH--VR----..D-SQSFKV..Y-....T--D-KTQ..-RL
HSD     1       TGWS--V---GGFL-QRIIR--VKEKELKEIR--..DKAFGPE--EEFSK.-.QN
CBR     1    MSS-IH-A-V--GNK-I-LAIVRD-CR-F-GDV--TARDVT-GQA-VQ--Q--G-SPR

         61       70        80        90       100       110       120
SPR    58 RSGLRVVRVPADLGAEAGLQQLLGALRELPRPKGLQ.RLLLINNAGSLGDVSKGFVDLSD
EDH    46 WEAA-ALAC-PGSLETLQ-DVRDSKSVAAA-ERVTEG-VDVLVCNAG--LLG.PLEA-GE
HSD    52 KTK-T-LEGDILDEPFLKRACQDVSVIIHTACIIDV.FGVTHRESIMNVNVKGTQLLLEA
CBR    59 FHQ-DIDDLQSIRALRDF-RKEY-G-DV-VNNA-IA.FKVADPTPFHIQAEVTMKTNFFG
```

Fig. 3. Comparison of the amino acid sequence of human sepiapterin reductase with those of human carbonyl reductase, estradiol 17ß-dehydrogenase, and 3ß-hydroxy-5-ene steroid dehydrogenase. Gaps (.) have been inserted to achieve a maximum homology. Identical amino acids with human sepiapterin reductase are indicated by hyphens (-). SPR, sepiapterin reductase; EDH, estradiol 17ß-dehydrogenase; HSD, 3ß-hydroxy-5-ene steroid dehydrogenase; CBR, carbonyl reductase.

alignment of their sequences. With this alignment, the sequence of residues 1-57 in human SPR show 37% identity (19 out of 52 positions compared) with that of residues 1-45 in human estradiol 17ß-dehydrogenase, and 63% similarity when conservative substitutions of amino acids are also taken into account. Similarly 25% identity (13 out of 52 positions) and 62% similarity with that of residues 1-51 in human 3ß-hydroxy-5-ene steroid dehydrogenase, 33% identity (19 out of 58 positions) and 71% of similarity with that of residues 1-58 in human carbonyl reductase are observed.

A putative pterin binding site and a NADPH binding consensus sequence on SPR are also located on the N-terminal region. The pattern of homology suggests that these molecules contain a similar core structure in the N-terminal portion containing the determinants for the enzymatic activity.

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