Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 6436–6440, August 1990
Genetics

Isolation and expression of rat liver sepiapterin reductase cDNA

(tetrahydrobiopterin/biopterin/pyruvoyltetrahydropterin/aromatic amino acid hydroxylase/molecular cloning)

BRUCE A. CITRON*, SHELDON MILSTIEN*, JOANNE C. GUTIERREZ†, ROBERT A. LEVINE‡, BRENDA L. YANAK*§, AND SEYMOUR KAUFMAN*¶

*Laboratory of Neurochemistry, Room 3D30, Building 36, and †Laboratory of Cell Biology, National Institute of Mental Health, National Institutes of Health, Bethesda, MD 20892; and ‡Laboratory of Molecular Neurobiology, Lafayette Clinic, 951 East Lafayette, Detroit, MI 48207

Contributed by Seymour Kaufman, June 7, 1990

ABSTRACT **Sepiapterin reductase (7,8-dihydrobiopterin: $NADP^+$ oxidoreductase, EC 1.1.1.153) catalyzes the terminal step in the biosynthetic pathway for tetrahydrobiopterin, the cofactor necessary for aromatic amino acid hydroxylation. We report here the isolation of a cDNA clone for rat liver sepiapterin reductase. The cDNA has been excised from a lambda vector and the DNA sequence was determined. The insert contains the coding sequence for at least 95% of the rat enzyme and is fused to the *Escherichia coli* β-galactosidase N-terminal segment and the *lac* promoter. The N-terminal region of the clone contains an extraordinarily high G+C content. The amino acid sequence deduced from the clone is in agreement with the size and composition of the enzyme and was matched to several tryptic peptide sequences. The enzyme encoded by the cDNA insert was shown to have sepiapterin reductase activity after expression in *E. coli*. Structural similarities were identified between this protein and several enzymes that should contain similar nucleotide and pteridine binding sites.**

The cofactor 5,6,7,8-tetrahydrobiopterin is an absolute requirement for the activity of the three aromatic amino acid hydroxylases phenylalanine hydroxylase, tyrosine hydroxylase, and tryptophan hydroxylase (1). The latter two hydroxylases catalyze rate-limiting steps in the biosynthesis of the neurotransmitters dopamine, norepinephrine, and serotonin. Whereas the aromatic amino acid hydroxylases have extremely limited tissue distribution, the biosynthetic enzymes for tetrahydrobiopterin, and tetrahydrobiopterin itself, are present in most cell types examined. This divergent tissue distribution may reflect broader roles for the cofactor than have yet been fully identified (2–4). Such widespread expression suggests that tetrahydrobiopterin may be important in some very basic cellular processes (4). In any case, the role of the cofactor in aromatic amino acid hydroxylation is critical to normal neurological function and several examples of cofactor deficiency disorders have been described (reviewed in ref. 5).

Sepiapterin reductase (7,8-dihydrobiopterin:$NADP^+$ oxidoreductase, EC 1.1.1.153) was first discovered in the chicken and rat liver by Matsubara *et al.* (6). The purification and characterization of sepiapterin reductase from rat erythrocytes has demonstrated it to be an acidic protein composed of two 28-kDa subunits (7). This enzyme is involved in one or both of the two final reductions of the *de novo* tetrahydrobiopterin biosynthetic pathway necessary to convert 6-pyruvoyltetrahydropterin to tetrahydrobiopterin (7–12), depicted in Fig. 1 (dashed line). There are two possible routes for single-step reductions of each of the carbonyl groups of 6-pyruvoyltetrahydropterin, with one pathway using only sepiapterin reductase for both reductions and the other using a different reductase to carry out the first reduction (9). In either case, the final carbonyl reduction to form tetrahydrobiopterin must be catalyzed by sepiapterin reductase.

In the present study, we have screened a rat liver cDNA library with a specific antibody that reacts positively to the enzyme band on Western blots (19). These clones were used to further characterize the enzyme and to begin to elucidate the functional sites of the enzymes in the tetrahydrobiopterin biosynthetic pathway.‖

MATERIALS AND METHODS

Screening. Sepiapterin reductase from rat erythrocytes was purified according to the procedure of Sueoka and Katoh (7). Antibodies to the enzyme were isolated and tested as described (19). All commercial reagents were used according to the manufacturers' recommendations. Antibody screens were carried out with the use of the Protoblot immunoscreening system (Promega) with our rabbit anti-rat erythrocyte sepiapterin reductase together with goat anti-rabbit IgG conjugated to alkaline phosphatase. A rat liver cDNA library (Stratagene) in the lambda ZAP II vector (20) containing 2×10^6 primary inserts was tested for the presence of sepiapterin reductase clones. Additional screening with a labeled DNA oligomer was carried out according to Church and Gilbert (21) conditions to identify full-length sepiapterin reductase inserts in phage vectors transferred *in situ* to Amersham Hybond N nylon membranes. Positive candidates with either procedure were purified through three subsequent rounds of isolation and then manipulated according to standard recombinant procedures (22, 23). DNA sequence analysis was carried out according to the dideoxynucleotide chain-termination method of Sanger *et al.* (24). The genotypes of the bacterial strains used have been described by Sambrook *et al.* (22).

Sequence Analysis. DNA and protein sequence comparisons were made with the aid of the University of Wisconsin Genetics Computing Group Programs version 6.1 (25). The data bases that were screened for similar sequences included the National Biomedical Research Foundation protein data base (version 35.0), which contained 12,476 protein sequences, and the GenBank (26) (version 62.0), and the European Molecular Biology Laboratory (27) (version 19.0) nucleic acid data bases, which held 31,232 different sequences. The sepiapterin reductase amino acid sequence was compared to the protein sequence data base and also to all of the theoretical translations of the nucleic acid entries with the aid of the programs FASTA and TFASTA, respectively, both with a word size of 2 (28). The sepiapterin reductase cDNA sequence was matched to the nucleic acid data bases by using FASTA and WORDSEARCH, each with a word size of 6.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

§Present address: Biology Department, Colgate University, Hamilton, NY 13346.
¶To whom reprint requests should be addressed.
‖The sequence reported in this paper has been deposited in the GenBank data base (accession no. M36410).

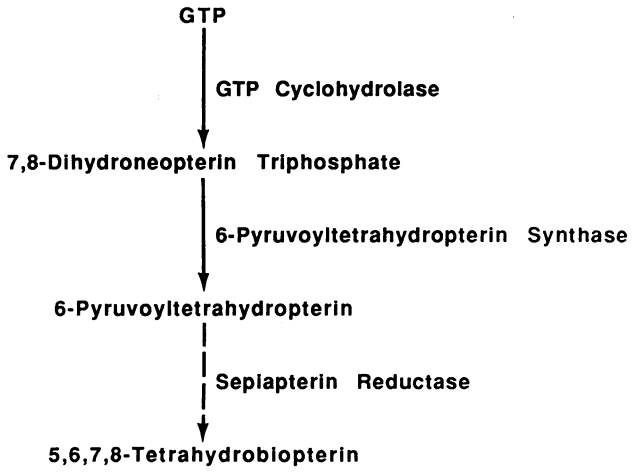

FIG. 1. The tetrahydrobiopterin biosynthetic pathway (11, 13–18). The last two reductions are carbonyl reductions, which may both be carried out by sepiapterin reductase or an alternative reductase may catalyze the first carbonyl reduction of 6-pyruvoyltetrahydropterin.

Peptide Sequencing. Purified sepiapterin reductase was reduced and carboxymethylated and then digested with trypsin [1% (wt/wt)]; the peptide fragments were separated by HPLC (reverse-phase Vydac C8, 15 × 0.46 cm) and collected. The tryptic peptides were sequenced on an Applied Biosystems 470A sequencer equipped with a 120A PTH analyzer. Peptides that yielded ambiguous data at all cycles were further separated by microbore reverse-phase HPLC (Brownlee C18, 30 × 2.1 mm) and the peaks were reanalyzed to produce distinct sequences.

Expression. Cultures (100 ml) of *E. coli* containing various plasmids were grown in 0.5% NaCl/1% tryptone/0.5% yeast extract/ampicillin (100 μg/ml) at 37°C with agitation until early logarithmic phase, induced with 0.5 mM isopropyl β-D-thiogalactopyranoside for 5 hr, harvested by centrifugation, and washed twice with 10 mM $MgSO_4$. The cells were broken open with four 5-sec bursts of sonication at 0°C and were assayed for sepiapterin reductase activity by measuring the disappearance of sepiapterin spectrophotometrically at 410 nm at 37°C in a reaction mixture containing 50 mM potassium phosphate (pH 6.5), 200 μM NADPH, and 50 μM sepiapterin (29).

RESULTS

Isolation of Sepiapterin Reductase cDNA. Rabbit anti-rat sepiapterin reductase antibody was used to screen 855,000 rat liver cDNA inserts in a λ library. Eight clones retained a positive signal after three rounds of screening and isolation. This frequency is 2- to 10-fold lower than the value predicted by the prevalence of the protein (0.01–0.2%) (7) and the likelihood that the insert should have been in-frame with the β-galactosidase peptide on the vector. Three of these cDNAs were excised to form phagemids for restriction site mapping, sequence analysis, and expression. The restriction patterns for two were indistinguishable and one of these, pBLY1, was sequenced and is depicted in Figs. 2 and 3.

pBLY1 contains a 787-base open reading frame linked to an *E. coli* β-galactosidase N-terminal fragment. The β-galactosidase translation frame in this plasmid is under the control of an *E. coli lac* promoter. When induced, this composite DNA molecule should produce a 298-residue fusion protein, the last 259 amino acids of which are derived from the cDNA insert. The molecular weight of a sepiapterin reductase subunit has been reported to be 27,500 (7). This value is consistent with a protein containing 230–270 amino acids and indicated that our clone was missing just a few N-terminal residues.

We searched for clones containing the complete N-terminal sequence by repeating the screening of the cDNA library with a 38-base DNA oligomer corresponding to the extreme 5′ end of the insert in pBLY1. Surprisingly, only one positive clone was obtained from these hybridizations. This clone perfectly matched the sequence of the oligomer and the sequence of the first several hundred bases of the insert in pBLY1. In addition, this clone contained 23 additional bases of sequence extending the 5′ end of the known sepiapterin reductase sequence. The new 5′ sequence showed that there were only three amino acids missing in the pBLY1 clone and that the N terminus of the sepiapterin reductase protein (not

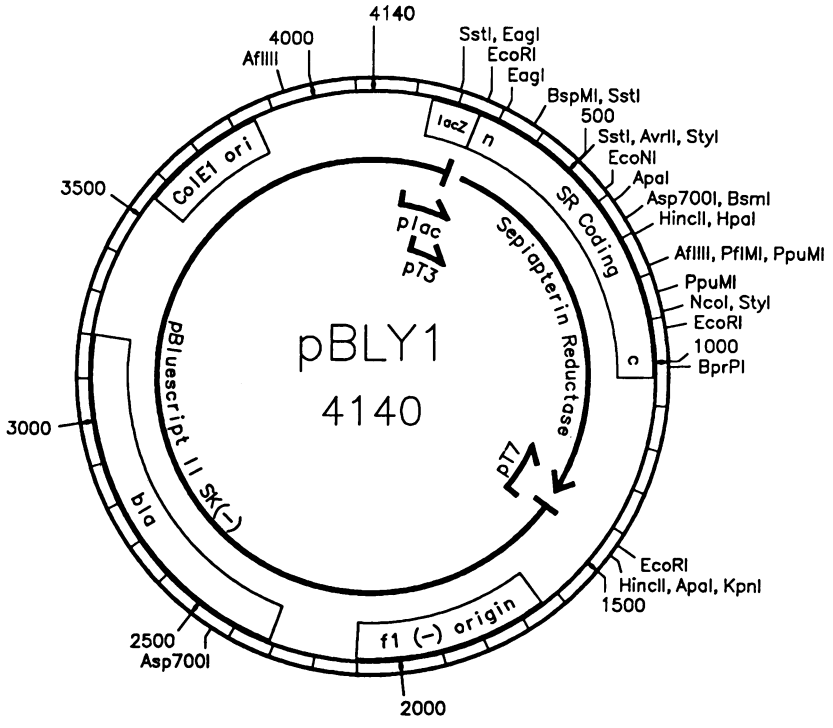

FIG. 2. The rat sepiapterin reductase clone. Plasmid pBLY1 contains 1171 bp of rat sepiapterin reductase cDNA including 777 bp of open reading frame linked to the N-terminal fragment of the β-galactosidase from the *E. coli lacZ* gene. Immediately upstream of this coding sequence is the promoter, p_{lac}, which is inducible with isopropyl β-D-thiogalactopyranoside. The plasmid contains a β-lactamase gene (*bla*), which confers resistance to ampicillin, a colE1 origin for double-stranded plasmid replication, a single-stranded phage (f1) origin for single-stranded replication, and two opposing promoters, p_{T3} and p_{T7}, which are useful for the synthesis of complementary RNA strands. All commercially available restriction enzyme sites that cleave pBLY1 once or twice and cut in the insert are shown in addition to the locations of the *Eco*RI and *Kpn* I sites, which are depicted to orient the cDNA in relation to the vector.

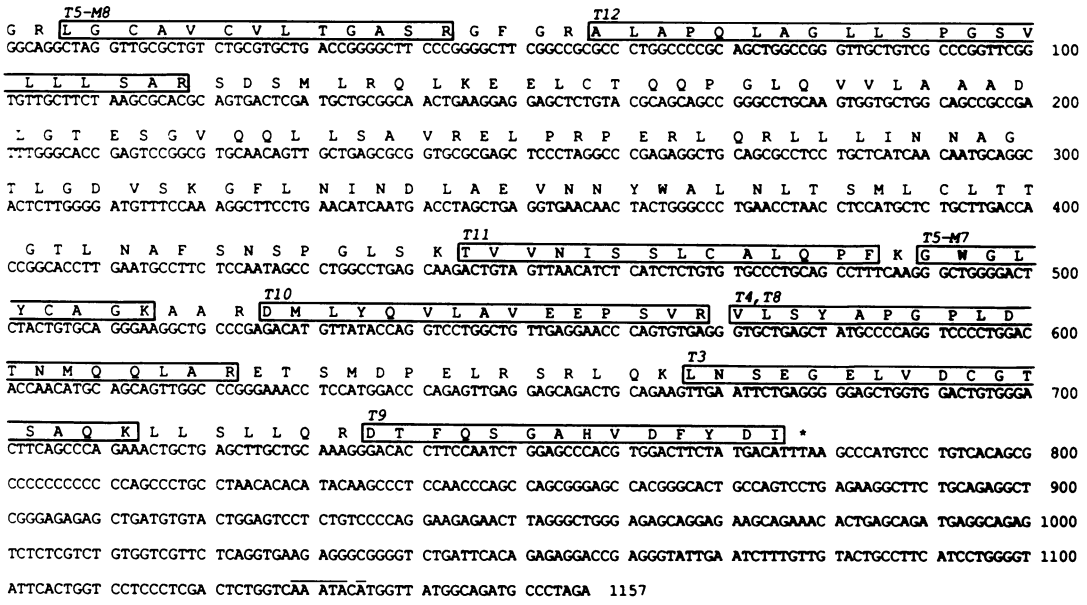

FIG. 3. The rat sepiapterin reductase cDNA insert. The 5′ end of the insert is immediately preceded by the remnant of an *Eco*RI linker, GAATTCC, and the 3′ end is followed by 14 A residues and the linker sequence GGAATTC. The linkers join the insert to the pBluescriptII SK(−) vector at its position 701 such that the reading frame is contiguous with the β-galactosidase N-terminal peptide. A putative polyadenylylation site is located at position 1157 and is possibly directed by the overscored signal sequence. Nine sequenced tryptic peptides are outlined and are in perfect alignment with the predicted protein with the following discrepancies: Peptide T4 seemed to have had a modified methionine at position 13 due to treatment prior to chromatography. This altered its mobility through the HPLC system to a position distinct from the unmodified peptide T8. T5-M7 and T5 originally comigrated but were finally separated on microbore HPLC.

shown in Fig. 3) is Met-Glu-Gly. This would give the enzyme a length of 262 amino acids. Other information indicated that this clone contained an internal rearrangement. Further upstream from the putative translation start site, the sequence in this clone suddenly switched to the noncoding strand in the middle of the sepiapterin reductase insert. Since this clone involved a rearrangement, these data were used only in the comparisons of the amino acid compositions.

The low yield of full-length clones could reflect an unusual structural feature such as a long dyad symmetry. This would inhibit the synthesis and also reduce the stability of long sepiapterin reductase cDNAs. An exceptional G+C content is present in the N terminus of the coding sequence in pBLY1 as shown in Fig. 4. Throughout the 1171-base-pair (bp) insert and the 2961-bp vector, the G+C content varies between 40% and 60%, but there is one 100-bp stretch at the 5′ end of the insert that is 70% G+C. *E. coli* selects against strong stem–loop structures and this feature may have eliminated the possibility of obtaining any intact, full-length structures for this gene.

Confirmation of the Amino Acid Sequence. To confirm that we have cloned the sepiapterin reductase cDNA, the previously purified protein was digested with trypsin and the peptides produced were purified by HPLC and sequenced.

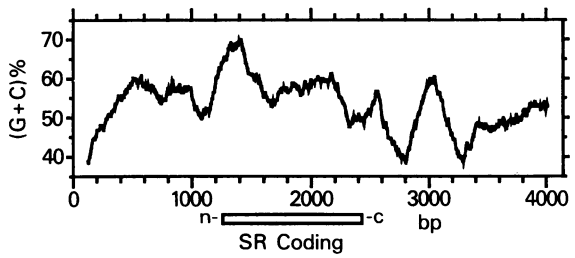

FIG. 4. G+C composition of pBLY1. The unusually rich G+C content at the N-terminal end of the sepiapterin reductase insert is shown by displaying the ratio through the entire plasmid beginning at pBLY1 coordinate 3140. Each point is the average G+C percentage for 250 consecutive bases.

No amino acids were found when the intact protein was sequenced, suggesting that the N terminus is blocked. The sequences of nine peptides were determined and all were in complete agreement with the amino acid sequence deduced from the cDNA as shown in Fig. 3. Comparison of the amino acid compositions for sepiapterin reductase translated from the cDNA sequence and those determined chemically are shown in Table 1 and these values were also in agreement.

Table 1. Amino acid composition of putative rat sepiapterin reductase deduced from the cDNA sequence and compared to amino acid analyses

		Amino acid analyses	
Amino acid	Sequence	This work	Sueoka and Katoh (7)
Asx	23	19.8	24.4
Glx	29	25.5	28.1
Ser	23	19.8	22.9
Gly	23	18.4	26.3
His	1	1.4	1.2
Thr	13	11.3	12.1
Ala	23	24.1	19.9
Arg	15	15.6	15.7
Pro	11	12.7	8.9
Tyr	5	5.7	4.9
Val	17	17.0	15.1
Met	6	5.7	5.6
Cys	7	4.2	5.7
Ile	4	4.2	4.6
Leu	47	48.2	38.5
Phe	6	5.7	6.5
Lys	7	7.1	7.8
Trp	2	1.9	13.9

The amino acid fractions for each composition analysis are normalized to a 262-residue polypeptide. The tryptophan value was determined after hydrolysis of the protein with methanesulfonic acid (30). Separate spectrophotometric determination of tryptophan yielded values 7-fold too high as did the spectrophotometric determination by Sueoka and Katoh (7).

Table 2. Sepiapterin reductase activity

Source	Sepiapterin reductase activity, $\mu mol \cdot min^{-1} \cdot mg^{-1}$
DH5 (pLNC101)	<0.01
DH5 (pBLY1)	1.1
DH5a (pBLY1)	1.4
HB101 (pBLY1)	1.7
RR1 (pBLY1)	1.3
Rat liver extract	0.0021
Pure rat erythrocyte	20

Specific activities of sepiapterin reductase in crude extracts of different *E. coli* strains induced for expression of the rat sepiapterin reductase insert on pBLY1 compared to the negative control plasmid, pLNC101 (a similar plasmid harboring a different cDNA), crude rat liver extract, and the pure form of the enzyme.

Expression. To test whether the sepiapterin reductase insert could produce a functional enzyme and to observe the consistency of expression between cultures, the pBLY1 clone was transformed into four different *E. coli* strains. The bacteria harboring the sepiapterin reductase cDNA or a negative control plasmid were grown under inducing conditions and crude extracts were assayed for sepiapterin reductase activity.

The specific activities observed in the *E. coli* containing the putative sepiapterin reductase cDNA averaged 650-fold higher than activities seen in crude rat liver extracts (see Table 2). Using the purified rat erythrocyte sepiapterin reductase specific activity as a standard, we calculated that ≈7% of the total *E. coli* protein in induced clones is sepiapterin reductase and we achieved a production rate slightly greater than 20 mg of sepiapterin reductase per liter of cultured *E. coli* (pBLY1) cells.

DISCUSSION

The sepiapterin reductase activity present in most tissues is probably due to the ubiquitous expression of the same gene (31). Although the importance of tetrahydrobiopterin synthesis in many tissues remains unclear, depressed synthesis may play a role in a variety of human diseases.

The sepiapterin reductase sequence present in pBLY1 does not share any significant global similarity to any protein or DNA sequence currently residing in the computer data bases. This indicates that no other members of a closely related evolutionary family have been reported in these data bases. However, small shared features could be buried in the statistical background of such analysis.

Wierenga *et al.* (32) have described a nucleotide binding consensus sequence of ≈30 amino acid residues. Sepiapterin reductase binds NADPH and the sequence was screened for a nucleotide binding site. There is a Gly-Xaa-Gly sequence that resembles the nucleotide binding consensus shown in Table 3. Although the match is not perfect, there are 7 strong similarities to the 11 consensus positions of the nucleotide binding site spanning 27 amino acids from position 11 to 37 (see Table 3). Furthermore, the match observed is located near the N terminus for the protein, the usual site for NADPH binding domains (32). The terminal position in the sepiapterin reductase putative nucleotide binding site contains a hydrophobic residue instead of the acidic residue of the NADH binding structures. This change is identical to the difference seen in glutathione reductase, another NADPH binding enzyme, and Wierenga *et al.* (32) note that the divergence in glutathione reductase is necessary to accommodate the additional phosphate group.

Another important feature in sepiapterin reductase is the pterin binding domain. Recently, anti-pterin binding site antibodies have demonstrated the structural relatedness between the pterin sites in sepiapterin reductase, dihydropteridine reductase, dihydrofolate reductase, and the aromatic amino acid hydroxylases (33). In that study, a single anti-idiotypic monoclonal antibody was able to bind to this group of enzymes, providing strong evidence for specific structures.

To help identify such potentially functional sites, we compared this protein sequence to the sequences of several enzymes (shown in Table 3) that interact with the same or similar pteridines. The aromatic amino acid hydroxylases are too closely related to reveal conserved sites for binding tetrahydrobiopterin. With the addition of the sepiapterin reductase sequence, it is now possible to compare and array sequences with related functionality (9, 34, 35) simultaneously to identify repeated sites. These protein sequences were examined graphically and statistically and there appear to be several sites that share statistically significant similarities.

Table 3 shows the strong local similarities between the N-terminal region of sepiapterin reductase and two sites in the middle and in the C-terminal regions of all five hydroxylases examined. This feature is a pentapeptide sequence (Ala-Gly-Leu-Leu-Ser) common to all of the hydroxylases, sepiapterin reductase, and (partially) to aldose reductase. There are at least two sites similar to Ala-Gly-Leu-Leu-Ser in

Table 3. Sequence similarities to sepiapterin reductase

Enzyme	Sequence	Position
Rat sepiapterin reductase	Ala-Gly-Leu-Leu-Ser	31
Rat and human phenylalanine hydroxylase	Ala-Gly-Leu-Leu-Ser	251, 350
Rat tyrosine hydroxylase	Ala-Ala-Leu-Leu-Ser	148
Rat tyrosine hydroxylase	Ala-Gly-Leu-Leu-Ser	251, 350
Quail tyrosine hydroxylase	Arg-Gly-Leu-Leu-Ser	290
Quail tyrosine hydroxylase	Ala-Gly-Leu-Leu-Ser	389
Human tyrosine hydroxylase type 4	Ala-Ala-Leu-Leu-Ser	178
Human tyrosine hydroxylase type 4	Ala-Gly-Leu-Leu-Ser	327, 426
Rat and rabbit tryptophan hydroxylase	Ala-Gly-Tyr-Leu-Ser	238
Rat and rabbit tryptophan hydroxylase	Ala-Gly-Leu-Leu-Ser	337
Rat aldose reductase	Ala-Thr-Leu-Leu-Ser	292
NADPH binding site consensus	TL–L–G–G––G–––L––L––––––L–L–D	
	| | | | | | |	
Rat sepiapterin reductase (position 11, Fig. 3)	TGASRGFGRALAPQLAGLLSPGSVLLLSA	

Paired and simultaneous comparisons between proteins that may contain functional domains yielded several sites that could be important for activity or regulation. The NAD binding consensus of Wierenga *et al.* (32) is simplified: T, basic or hydrophilic; L, small and hydrophobic; G, glycine; D, acidic.

all of the known aromatic amino acid hydroxylases. Since this sequence is found only once in sepiapterin reductase, the duplications might indicate the presence of two pterin binding sites in the hydroxylases. Significantly, the sequence Ala-Gly-Leu-Leu-Ser is extremely rare in known protein sequences and occurs in only six other proteins in the NBRF data base.

In addition, there are broad regions of similarity between human dihydrofolate reductase, human and rat aldose reductase [an enzyme that can catalyze the NADPH-mediated reduction of 6-pyruvoyltetrahydropterin (36)], and sepiapterin reductase. The amino acid sequence from positions 198–257 (in Fig. 3) shares 20–37% identity to the aromatic amino acid hydroxylases, human aldose reductase, and human dihydrofolate reductase for lengths of 20–45 residues. These positions may also be candidates for participation in pterin binding.

It is significant that the C-terminal domain of sepiapterin reductase shares similarity to several other pterin binding enzymes. This region of the protein also contains the most hydrophilic stretch in a predominantly hydrophobic enzyme. Because the pterin substrates are positively charged at neutral pH, they would be expected to have an affinity for hydrophilic residues and it is possible that this is the region of the protein involved in pterin binding. Since the enzyme produced by pBLY1 is active, it should be possible to modify these residues by site-directed mutagenesis to test this hypothesis.

Final identification of the structural motifs in the tetrahydrobiopterin biosynthetic enzymes awaits more sequences for comparison and site-directed mutagenesis studies. However, the first step toward testing structure–function relationships has begun with the cloning and expression of sepiapterin reductase in an organism readily amenable to biochemical genetics.

We thank Dr. Michael J. Brownstein for providing synthetic DNA oligomers, Dr. Peter S. Backlund for amino acid analyses, Michele R. Belecanech for superb technical assistance, Margot L. Gibson for expert assistance with DNA sequencing, and Dr. Robert Pohlman for a critique of the manuscript.

1. Kaufman, S. (1974) in *Amino Acids in the Brain*, eds. Wolstenhome, G. E. W. & Fitzsimons, D. W. (Elsevier, New York), pp. 85–115.
2. Kaufman, S. (1986) in *Chemistry and Biology of Pteridines*, ed. Pfleiderer, W. (de Gruyter, New York), pp. 185–200.
3. Milstien, S. (1987) in *Unconjugated Pterins and Related Biogenic Amines*, eds. Curtius, H.-Ch. & Blau, N. (de Gruyter, New York), pp. 49–65.
4. Tanaka, K., Kaufman, S. & Milstien, S. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 5864–5867.
5. Kaufman, S. (1987) *Top. Neurochem. Neuropharmacol.* **142**, 1–28.
6. Matsubara, M., Katoh, S., Akino, M. & Kaufman, S. (1966) *Biochim. Biophys. Acta* **122**, 202–212.
7. Sueoka, T. & Katoh, S. (1982) *Biochim. Biophys. Acta* **717**, 265–271.
8. Kaufman, S. (1963) *Proc. Natl. Acad. Sci. USA* **50**, 1085–1093.
9. Milstien, S. & Kaufman, S. (1989) *J. Biol. Chem.* **264**, 8066–8073.
10. Milstien, S. & Kaufman, S. (1989) *Biochem. Biophys. Res. Commun.* **165**, 845–850.
11. Curtius, H.-Ch., Heintel, D., Ghisla, S., Kuster, T., Leimbacher, W. & Niederwieser, A. (1985) *Eur. J. Biochem.* **148**, 413–419.
12. Smith, G. K. (1987) *Arch. Biochem. Biophys.* **255**, 254–266.
13. Yim, J. J. & Brown, G. M. (1976) *J. Biol. Chem.* **251**, 5087–5094.
14. Fukushima, K., Richter, W. E., Jr., & Shiota, T. (1977) *J. Biol. Chem.* **252**, 5750–5755.
15. Switchenko, A. C. & Brown, G. M. (1985) *J. Biol. Chem.* **260**, 2945–2951.
16. Masada, M., Akino, M., Sueoka, T. & Katoh, S. (1985) *Biochim. Biophys. Acta* **840**, 235–244.
17. Milstien, S. & Kaufman, S. (1985) *Biochem. Biophys. Res. Commun.* **128**, 1099–1107.
18. Smith, G. K. & Nichol, C. A. (1986) *J. Biol. Chem.* **261**, 2725–2737.
19. Levine, R. A., Kapatos, G. K., Kaufman, S. & Milstien, S. (1990) *J. Neurochem.* **54**, 1218–1224.
20. Short, J. M., Fernandez, J. M., Sorge, J. A. & Huse, W. D. (1988) *Nucleic Acids Res.* **16**, 7583–7600.
21. Church, G. M. & Gilbert, W. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 1991–1995.
22. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
23. Ausubel, F. M., Brent, R., Kinston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K., eds. (1987) *Current Protocols in Molecular Biology* (Wiley, New York).
24. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
25. Devereux, J., Haeberli, P. & Smithies, O. (1984) *Nucleic Acids Res.* **12**, 387–395.
26. Bilofsky, H. S., Burks, C., Fickett, J. W., Goad, W. B., Lewitter, F. I., Rindone, W. P., Swindell, C. D. & Tung, C. S. (1986) *Nucleic Acids Res.* **14**, 1–4.
27. Hamm, G. H. & Cameron, G. N. (1986) *Nucleic Acids Res.* **14**, 5–10.
28. Pearson, W. R. & Lipman, D. J. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 2444–2448.
29. Katoh, S. (1971) *Arch. Biochem. Biophys.* **146**, 202–214.
30. Simpson, R. J., Neuberger, M. R. & Liu, T. Y. (1976) *Anal. Biochem.* **254**, 1936–1940.
31. Ferre, J. & Naylor, E. W. (1987) *Biochem. Biophys. Res. Commun.* **148**, 1475–1481.
32. Wierenga, R. K., Terpstra, P. & Hol, W. G. (1986) *J. Mol. Biol.* **187**, 101–107.
33. Jennings, I. & Cotton, R. (1990) *J. Biol. Chem.* **265**, 1885–1889.
34. Sueoka, T. & Katoh, S. (1985) *Biochim. Biophys. Acta* **843**, 193–198.
35. Milstien, S. & Kaufman, S. (1984) in *Biochemical and Clinical Aspects of Pteridines*, eds. Pfleiderer, W., Wachter, H. & Curtius, H.-Ch. (de Gruyter, New York), Vol. 3, pp. 53–59.
36. Steinerstauch, P., Wermuth, B., Leimbacher, W. & Curtius, H.-Ch. (1989) *Biochem. Biophys. Res. Commun.* **164**, 1130–1136.