The 1.25 Å crystal structure of sepiapterin reductase reveals its binding mode to pterins and brain neurotransmitters

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Sepiapterin reductase catalyses the last steps in the biosynthesis of tetrahydrobiopterin, the essential co-factor of aromatic amino acid hydroxylases and nitric oxide synthases. We have determined the crystal structure of mouse sepiapterin reductase by multiple isomorphous replacement at a resolution of 1.25 Å in its ternary complex with oxaloacetate and NADP. The homodimeric structure reveals a single-domain α/β-fold with a central four-helix bundle connecting two seven-stranded parallel β-sheets, each sandwiched between two arrays of three helices. Ternary complexes with the substrate sepiapterin or the product tetrahydrobiopterin were studied. Each subunit contains a specific aspartate anchor (Asp258) for pterin-substrates, which positions the substrate side chain C1'-carbonyl group near Tyr171 OH and NADP C4'. The catalytic mechanism of SR appears to consist of a NADPH-dependent proton transfer from Tyr171 to the substrate C1' and C2' carbonyl functions accompanied by stereospecific side chain isomerization. Complex structures with the inhibitor N-acetyl serotonin show the indoleamide bond such that both reductase and isomerase activity for pterins is inhibited, but reaction with a variety of carbonyl compounds is possible. The complex structure with N-acetyl serotonin suggests the possibility for a highly specific feedback regulatory mechanism between the formation of indoleamines and pteridines in vivo.

Keywords: crystal structure/N-acetyl serotonin/SDR family/sepiapterin reductase/tetrahydrobiopterin

Introduction

Tetrahydrobiopterin (BH₄) is a multifunctional co-factor for phenylalanine, tyrosine and tryptophan hydroxylases, which catalyse the initial steps in phenylalanine degradation in the liver, and are the rate-limiting steps in the biosynthesis of the neurotransmitters, catecholamines and indoleamines in the brain.

A function to promote release of dopamine, serotonin and noradrenaline from the striatal and cortical nerve terminals has also been proposed for BH₄ (Mataga et al., 1991). Serotonin (5-hydroxytryptamine; 5-HT) and its derivatives are neurotransmitters present in brain or pituitary gland, regulating a great number of physiological mechanisms such as sleep, appetite, thermoregulation, control of pituitary secretions and behaviour (McGeer et al., 1987). BH₄ has furthermore an essential role in the biosynthesis of nitric oxide (NO) (Lowenstein and Snyder, 1992; Marletta, 1994) as an allosteric activator of nitric oxide synthase (NOS) and seems to be necessary for catalytic turnover involving a redox-function of the co-factor (Hemmens and Mayer, 1996). Recently, it was shown that an increase in BH₄ biosynthesis in a pancreatic B-cell line (INS-1) is followed by enhanced NO production and subsequently, inhibition of insulin secretion (Labranche et al., 1997). BH₄ regulates human melanogenesis by forming a stable complex with the α-melanocyte-stimulating hormone (Schallreuter et al., 1994). Finally, BH₄ is known as an essential co-factor for alkylglyceryl monoxygenases (Pfleger et al., 1967).

Reduced levels of BH₄ in the brain and cerebrospinal fluid are associated with several neuropsychiatric diseases such as Parkinson’s disease, Alzheimer’s disease, depression and dystonia (Lovenberg et al., 1979; Curtius et al., 1983). In atypical phenylketonuria (PKU), BH₄ deficiency results in neurological disorders as a result of decreased biosynthesis of brain catecholamines and serotonin. BH₄ is involved in proliferation and growth regulation of erythroid cells. Partial depletion of BH₄ in a murine erythroleukaemia cell line caused inhibition of cell growth (Tanaka et al., 1989).

The complex organic chemistry involved in de novo formation of BH₄ from GTP is catalysed by only three enzymes. The first committed step is catalysed by GTP cyclohydrolase I (EC 3.5.4.16; CYH) conducive to the formation of dihydrobiopterin triphosphate. This intermediate is transformed to 6-pyruvoyl tetrahydropterin (6-PHP₄) by 6-pyruvoyl tetrahydropterin synthase (EC 4.6.1.10; PTPS, PHP₄S). Finally, sepiapterin reductase (EC 1.1.1.153; SR) reduces this diketo compound in an NADPH-dependent step to BH₄. Recently, the crystal structures of Escherichia coli CYH (Nar et al., 1995a,b) and rat liver PTPS (Nar et al., 1994; Bürgisser et al., 1995) were solved by isomorphous replacement techniques (Auerbach and Nar, 1997). The crystal structure of mouse sepiapterin reductase (mSR) completes the structural analysis of all three enzymes involved in the BH₄ pathway (Figure 1). The biosynthetic pathway of BH₄ includes minimally these three enzymes; the participation of a fourth enzyme, aldose reductase, is suggested but still controversial. However, it was shown that aldose reductase is not important for BH₄ biosynthesis in liver (Milstein and Kaufman, 1989). Besides the de novo biosynthesis of BH₄, SR is also known to be involved in the pterin salvage pathway catalysing the conversion of sepiapterin.
Fig. 1. The complete pathway of the *de novo* biosynthesis of BH₄. The cofactor BH₄ is synthesized by only three enzymes, namely CYH, PTPS and SR. The crystal structure of E.coli CYH was recently solved by single isomorphous replacement and averaging techniques. The enzyme complex, a decamer consisting of a pentamer of tightly associated dimers, has perfect D₅ symmetry and is doughnut-shaped with dimensions of 65×100 Å. The MIR-solved crystal structure of rat PTPS shows a hexameric enzyme composed of a dimer of trimers with D₃ symmetry. Each trimer forms a 12-stranded antiparallel β-barrel, enclosing a basic pore with 6–12 Å diameter. The crystal structure of mSR completes the structural analysis of all three enzymes involved in BH₄ biosynthesis, providing the essential information for the interpretation of the complex biochemical regulation of this pathway.

to dihydrobipterin (BH₂) which is transformed by dihydrofolate reductase to BH₄ (Nichol et al., 1983). Furthermore, a regeneration system for the cofactor is known involving pterin-4a-carbinolamine dehydratase (PCD) (Endrizzi et al., 1995; Ficner et al., 1995) and dihydropteridine reductase (DHPR) (Varughese et al., 1992).

The full complement of the three BH₄-biosynthesizing enzymes can be found in significant amounts in many tissues of various species (Smith and Nichol, 1984). The richest sources of SR are erythrocytes, liver and brain. Katoh and co-workers (1982) have obtained evidence for a regulation mechanism of the BH₄ pathway by feedback.
inhibition of the activity of SR in rat brain by a catecholamine and an indoleamine.

SRs from mouse, rat and human origin have been cloned and sequenced (Citron et al., 1990; Ichinose et al., 1991; Ota et al., 1995) revealing a sequence identity of the mouse SR with rat and human enzymes of 88% and 74% (94% and 88% sequence similarity) (Figure 2A).

Here we describe a crystallographic structure analysis of mouse SR. All structures are complexes with the natural cofactor NADP, which was added during purification and crystallization. Surprisingly, recombinantly expressed protein contained oxaloacetate bound to the active site. The ternary complex structure of mouse SR with NADP and oxaloacetate was refined to a resolution of 1.25 Å. In order to investigate the catalytic mechanism of the enzyme, ternary complex structures either with the substrate, sepiapterin, or the product, tetrahydrobiopterin, have been analysed. Inhibitory complex structures were determined as quaternary complex with N-acetyl serotonin (NAS) and oxaloacetate (OAA), and as ternary complex structures with either NAS or noradrenaline.

Results and discussion

Overall structure of the SR

SR is a homodimer with 261 amino acids per monomer, each forming a single domain α/β-structure. A seven-stranded parallel β-sheet in the centre of the molecule is
sandwiched by two arrays of three α-helices (αC, αB, αG and αD, αE, αF). Six strands of them constitute a classical dinucleotide-binding motif (Rossmann et al., 1975) composed of βαβ units (Figure 2B). The association of two SR monomers to a dimer leads to the formation of a four-helix bundle by helices αE and αF of each monomer, stabilized by numerous hydrogen bonds and two charged interactions involving Asn116 and Asn141.

Fig. 3. Active site of mSR in complex with substrate sepiapterin (SPT) and cofactor NADP (NAP). Anchoring of sepiapterin with its purine moiety to Asp258 positions its C1’ carbonyl function in direct proximity to NADP and Tyr171, clearly indicating the latter as the central catalytic site for hydrogen transfer to the carbonyl function of the substrate.
from each monomer. Exceptionally, the two parallel β-sheets of the dimer are in an anti-parallel orientation enclosing an angle of 90°. The two substrate pockets are opened to the same side of the molecule, each formed by elongation of the three loops between strand βD and helix αE, between βE and αF, and between βF and αG. The latter insertion contains two additional helices αFG1 and αFG2 (Figure 2).

From both amino acid sequence and three-dimensional structure, SR can be unequivocally assigned to the family of short-chain dehydrogenases/reductases (SDR). Although sequence comparison of these enzymes shows sequence identities between 12% and 35% and their substrates are very different, they share a common tertiary structure. They all use either NAD(H) or NADP(H) as the cofactor and contain a strictly conserved Tyr-X-X-X-Lys sequence motif (Ghosh et al., 1994).

**Active site for reductase and isomerase activity**

The presence of the nicotinamide ring of the bound cofactor NADP already indicates the location of the active site of SR, which is situated at the C-terminal end of the β-strands. The acceptor site for the substrate contains a 15 Å-deep pocket that is ideally suited to receive pterin and small carbonyl substrates. The active site cavity is formed by the hydrophobic residues Leu105, Leu159, Tyr165, Trp168, Tyr171, Met206 and Cys160. The crystal structure of SR in ternary complex with NADP and the substrate, sepiapterin, clearly shows the pterin substrate positioned in the active site anchored with its guanidino moiety to Asp258. This positions the pterin moiety with its side chain C1'-carbonyl function in direct proximity to Tyr171 OH and NADP C4'. The crystal structures clearly show Tyr171 as the central active site residue in an orientation for optimal proton transfer to the carbonyl functional groups of the substrate. Furthermore, the nicotinamide group is co-planar with the side-chain carbon atoms for optimal hydride transfer to the carbonyl group (Figure 3). The electron density at the C2'-position in the ternary complex of SR with sepiapterin and NADP appears planar, thus indicating that sepiapterin is oxidized to the diketo compound in the soaked crystals (Figure 4A). The crystal structure of the ternary complex of the enzyme with NADPH and BH4, the product of the catalysed reaction, confirms this orientation of the side chain also for the bound product (Figure 4B).

Three residues can be regarded as a central feature in the active site of SR for both reductase and isomerase activity. The two basic residues Lys175 and Arg178 may facilitate the proton transfer from the hydroxyl function.

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**Fig. 4.** Electron densities for the substrate sepiapterin (SPT), the product tetrahydrobiopterin (BH4), the cofactor NADP, and the inhibitors N-acetyl serotonin (NAS) and oxaloacetate (OAA). (A) Stereo diagram showing the substrate sepiapterin in the enzyme-bound conformation placed in its 3σ contoured Fo-Fc density refined to 1.95 Å resolution and (B) the product tetrahydrobiopterin refined to 2.6 Å resolution. (C) Stereo view of the cofactor NADP placed in its 1σ contoured 2Fo-Fc density refined to 1.25 Å resolution. (D) Stereo view of the quaternary complex structure of SR with the two inhibitors OAA and NAS. NAS, anchored by Asp258, is a specific competitive inhibitor only for pterin substrates, while OAA bound to Tyr171 is probably an inhibitor for pterin- and non-pterin carbonyl compounds.
of Tyr171 to the substrate's carbonyl oxygen by stabilizing the resulting tyrosinate. The guanidinium group of Arg178 is in hydrogen bond distance to the side-chain amide groups of the three Asn residues, Asn100, Asn128 and Asn155, which are conserved at least within SR, carbonyl reductase, and Drosophila alcohol dehydrogenase.

From the crystal structure, a central role can also be proposed for residue Ser158, which is located in hydrogen bond distance to the substrate N5 atom and the C1'-side chain oxygen. Ser158 is presumably involved in stabilizing the position of the substrate, according to results from 7α-hydroxy steroid dehydrogenase (HSDH), mouse lung carbonyl reductase (MLCR) (Tanaka et al., 1996a,b), and 3β/17β-HSDH (Oppermann et al., 1997). Ser158 is close to the residues Cys160 and Cys172, providing possible additional proton sources. The conserved cysteine residues are in the reduced state in all analysed crystals, independent of the presence of dithiothreitol. Conformational changes by substrate-binding as seen in a so-called substrate-binding loop in the structure of 7α-HSDH (Tanaka et al., 1996b), which aligns with the mSR region Asn205 to Asp229 (helices αFG1 and αFG2), do not occur in mSR. This part seems rather rigid with temperature factors below 20 Å² in mSR.

As confirmed by several complex structures, the active site is highly accessible in the crystals of SR by opening the substrate-binding pockets towards honeycomb-like cavities. Major structural changes induced on binding of active site ligands are not observed.

**Coenzyme-binding mode**

The MIR-phased electron density already clearly identified the functional cofactor binding site of the enzyme. The adenine moiety of NADP is bound sandwiched between the side chains of Arg43, Leu71 and Leu127, and is anchored via hydrogen bonds to Asp70 and Leu71. On the basis of the first crystal structure at very high resolution of an NAD(P) co-factor in the enzyme-bound state, we are able to determine not only the conformation of the co-factor with high precision, but also the stereochemistry for the optimal hydride transfer reaction. The bound NADP molecule is in an extended conformation with the adenine ring in antit and the nicotinamide ring in syn conformation (Figure 4C), quite similar to the structures of HSDH and DHRP. The distance between C6 of the adenine and C2 of the nicotinamide is 14.2 Å for mSR, which is close to the values of 14.4 Å for 7α-HSDH, 14.6 Å for 3α,20β-HSDH and 15.1 Å for DHRP (Tanaka et al., 1996b). The syn conformation for the nicotinamide ring allows a B-face hydride transfer reaction. Both ribose rings have a C2'-endo puckering. The distances between Tyr171 OH and the carbonyl oxygen atom and between the C-4 of the nicotinamide and the carbonyl carbon atom are 2.7 Å and 3.2 Å, respectively. The stacking interaction of Arg43 with the adenine ring stabilizes the position of this residue similar to glutathione reductase reported by Pai et al. (1988). Arg43 is conserved in all known members of the SDR family which prefer NADPH (Tanaka et al., 1996a) instead of NADH.

**Catalytic mechanism**

SR catalyses the NADPH-dependent reduction of various monocarbonyl compounds including ketones and aldehydes. SR is capable of catalysing the NADPH-dependent reduction of both side-chain keto groups of 6-pyruvoyl tetrahydropterin (PH₄) to produce BH₄ with proper stereospecificity (Katoh and Sueoka, 1984; Smith, 1987). Asp258 seems essential in determining the substrate-binding specificity and anchors pterin derivatives for the reduction at their monoketo or diketo side chains.

Mouse SR represents the first member of the SDR family in complex with a substrate and a product. Thus, the complex structures of SR set the stage for the detailed scenario of the catalytic reaction. The catalytic mechanism of SR appears to consist of a stereospecific transfer of a hydride ion from the exposed C-4 of the nicotineamide to the carbon of the carbonyl group of the substrate and abstraction of a proton from Tyr171 by the incipient negatively charged carbonyl oxygen. Lys175 stabilizes the position and orientation of the nicotineamide nucleoside moiety of the bound cofactor NADP through a bifurcated hydrogen bond to both the 2'-hydroxyl and 3'-hydroxyl group of the ribose group comparable with carbonyl reductase reported by Tanaka et al. (1996a). After side chain motion, Lys175 is able to stabilize the position of the hydroxyl group of the tyrosine base catalyst by concomitantly lowering its pKₐ.

A common mechanism within the SDR family was suggested to involve a tyrosine and a lysine residue, both conserved within short-chain dehydrogenases/reductases (Jörnvall et al., 1981; Ghosh et al., 1994).

The first isolated intermediate in the reduction of PH₄ by SR is 1'-hydroxy-2'-oxo tetrahydropterin (Smith, 1987); this is also the compound produced in the reverse reaction from BH₄ (Curtius et al., 1985). These data indicate that the enzyme preferentially reduces the 1' keto group, consistent with the high reactivity of the enzyme toward pterin and lactoyl tetrahydropterin as compared with 1'-hydroxy-2'-oxo tetrahydropterin. There are two possible pathways for complete reduction of the diketo substrate. First, after reduction of the C1' carbonyl function and side-chain reorientation towards Tyr171 OH and NADPH C4'N, caused by the change in hybridization of the C1' carbon atom, the second reduction step could take place at the C2' carbonyl function. The second, stereochemically more attractive, alternative is that the reduction only occurs at the C1' carbon atom and the second carbonyl function is reduced after isomerization at the C1' and C2' via an enediol intermediate which is stereospecifically stabilized by the Tyr171 anion. Evidence for an enediol form of pterin was obtained by NMR studies (Iwanami and Akino, 1975). This isomerization is also catalysed in the absence of NADPH in the conversions of 6-lactoyl H₄ pterin and 6-lactoyl 7,8-BH₄ (sepiapterin) into the 6'-1'-hydroxy-2'-oxopropyl pterins (Katoh and Sueoka, 1987). The tetrahydro isomers were observed as the monoketo intermediates in the de novo BH₄ biosynthesis (Smith, 1987). There are other examples of isomerization of a keto-hydroxy group before reduction to the diol: reduction of camphorquinone by 3α-HSDH (Boutin, 1986) and in the reduction of cortisol to cortol in corticosteroid metabolism (Monder and Bradlow, 1980). Thus, the initial step of the conversion of PH₄ to BH₄ is the NADPH-dependent reduction at the side-chain C1'-keto function conducive to the formation of 1'-OH-2'-oxopropyl PH₄ (Figure 5; reaction 1). Internal rearrangement of
Inhibition of SR

Competitive inhibition of SR seems feasible for pterin- and non-pterin substrates. Anchoring of an analogue to Asp258 inhibits the reduction and isomerization of pterin derivatives, whereby the catalysis of small non-pterin substrates remains possible. However, binding of an inhibitor, like oxaloacetate, directly to the catalytic centres of the active site, Tyr171 and NADP C4’N, disables the enzyme for both pterin- and non-pterin substrates (Figure 4D).

Katoh et al. (1982) reported that N-acetyl serotonin (NAS) is a potent inhibitor of partially purified rat brain and homogeneous rat erythrocyte SR (K_i = 0.2 and 0.17 μM, respectively). These data suggested that NAS binds at the substrate site and Smith et al. (1992) proposed the N-acetyl group and indole hydroxyl function of NAS critical for binding. The binding mode of NAS is shown in Figure 6, derived from a 2.1 Å refined complex structure of mSR with NAS. The high specificity of SR for this inhibitor is achieved by anchoring the indoleamine with its hydroxyl function to Asp258 like the substrate and with its N-acetyl function to Tyr165 via a π-electron interaction. The presumed role of the aromatic ring is also indicated by a reorientation of the Tyr side chain after NAS binding. A sequence comparison shows a corresponding Phe residue in the enzymes from rat and human origin. Furthermore, anchoring via the substrate N-acetyl function explains the strongly reduced K_i value with regard to serotonin. The inhibitor NAS is bound with its indole moiety to the hydrophobic bottom of the pterin binding
site formed by Leu159, Leu226 and Tyr260, and with its acetyl group into a hydrophobic side pocket of the active site formed by the side chains of the residues Tyr165, Thr168, Leu219 and Leu223. Thereby, the phenyl ring of NAS binds in the pyrimidine pocket of the enzyme with its 5-hydroxyl aligned at the pyrimidine 3-position. The same binding mode can be proposed for melatonin, however, with a sterically less favourable methoxy group replacing the hydroxyl position of NAS.

In summary, SR evaluates two very specific binding motifs for competitive inhibition of the enzyme (versus serotonin): the Asp258 anchor, embedded in a hydrophobic pterin binding site, and Tyr165, forming a nitrogen anchor within a hydrophobic side pocket of the active site close to Asp258 and Tyr171. In vivo, only NAS seems to be optimally able to occupy both of these two motifs by forming a strong inhibitory complex with SR ($K_i = 0.12 \mu M$). Neither serotonin ($K_i = 2.3 \mu M$), an intermediate in the indoleamine pathway just before NAS and lacking the N-acyl group, nor melatonin ($K_i = 30 \mu M$), directly after NAS with an additional methoxy group at the aromatic hydroxyl position of NAS, can achieve this tight binding mode. Because the acetyl group of NAS is not completely filling the hydrophobic pocket around Tyr165, elongation of the inhibitor’s side chain should increase the binding ability. This is realized in the case of synthetic inhibitors, like N-chloro- and N-methoxy-acetyl serotonin, which are one of the tightest inhibitors known so far ($K_i$ value = 0.006 $\mu M$, respectively 0.008 $\mu M$).

Based on the crystal structure of the ternary complex with NAS and OAA, it should be possible to design synthetic inhibitors, which bind to three specific sites within the active site of SR, namely Asp258, the hydrophobic pocket near Tyr165, and Tyr171.

Oxaloacetate is already bound to native crystals of the recombinant protein expressed in E.coli and was crystallographically identified. Its $\alpha$-ketio terminal carboxylate group bound to Tyr171 imitates the C1-carbonyl function of the substrate side chain. The inhibitory role of oxaloacetate requires further investigations by kinetic studies. Oxaloacetate can be replaced by soaking the protein with serotonin. It is remarkable that malate dehydrogenase (MDH), which catalyses the regeneration of oxaloacetate in the final reaction step of the citric acid cycle, shows significant overall structural homology with SR.

In order to confirm the binding mode of NAS to the active site also without OAA, the latter was removed from the protein using serotonin and charcoal before crystallization. The analysed ternary complex structure of the enzyme with NAS and NADP exactly confirmed the binding mode found in the quaternary complex with NAS, OAA and NADP.

**Comparison with other structures**

*SDR members and other single-domain oxidoreductases.*

More than 50 different enzymes have been characterized as belonging to the SDR family (Jörnvall et al., 1995). In contrast, the three-dimensional structures have been elucidated only for four enzymes of this family: 3α,20β-hydroxy steroid dehydrogenase (3α,20β-HSDH) (Ghosh et al., 1994), dihydropteridine reductase (DHPR) (Varughese et al., 1992), 7α-hydroxy steroid dehydro-

genase (7α-HSDH) (Tanaka et al., 1996b), and mouse lung carbonyl reductase (MLCR) (Tanaka et al., 1996a).

Despite the fact that the amino acid sequence identity among these enzymes is <30%, they share a similar tertiary structure with SR. SR, DHPR (122 Ca atoms aligning within 3.0 Å with mSR) and 17β-HSDH (159 Ca atoms) are dimeric enzymes, while MLCR (183 Ca atoms), 7α-HSDH (174 Ca atoms) and 3α,20β-HSDH (171 Ca atoms) are tetrameric. The recently solved crystal structure of the isoniazid target of *Mycobacterium tuberculosis* (erythrocromobacter protein reductase; InhA) (Dessen et al., 1995), shows a single-domain oxidoreductase not belonging to the SDR family, however, revealing 146 Ca atoms aligning to mSR.

**The three enzymes of the pathway of \(BH_4\) biosynthesis.**

The topology of the structure of SR is different from the first two enzymes of the \(BH_4\) pathway, which show a similar subunit fold by comparing the C-terminal domain of CYH with RPTS (Nar et al., 1995b). The most striking difference between SR and the other two enzymes in the pathway is a parallel β-sheet of SR instead of the anti-parallel, barrel-forming β-sheets of CYH and RPTS. However, SR shows the same architecture as CYH and RPTS with regard to a β-sheet flanked by the two helices αB and αG (mSR). The complex structures of the three enzymes with their natural substrates establish a common binding motif for pterins quite similar to the GTP binding motif in small GTP binding proteins (Bourне et al., 1991). Despite the varying natyure of the three substrates, which are subsequently processed in the \(BH_4\) biosynthetic pathway (Figure 1), the guanidino function is specifically recognized by the active sites of the three enzymes by either a Glu or Asp residue. The affinity between carboxylic acids and the 2-amino pyrimidine moiety has been demonstrated in several studies (Etter and Adsdon, 1990). The residues Glu152 (E.coli CYH), Glu107 (rat liver PTPS), and Asp258 (mSR), respectively, form hydrogen bonds with both of its carboxylate oxygens to the substrate nitrogens N-2 and N-3. Supporting this anchor, a preceding peptide bond between Val150 and Gln151 in CYH, Thr105 and Thr106 in PTPS, or Ser158 and Leu159 in SR, forms a hydrogen bond with its amide hydrogen to the C=O oxo group of the purine. There is no sequence identity among the three enzymes.

**Significance and implications**

According to the kinetic results, almost all of the activity of brain or erythroid SR is inhibited by NAS or melatonin. Both indoleamines are synthesized in the pineal gland via the rate-limiting and \(BH_4\)-dependent step catalysed by tryptophan hydroxylase. The role of melatonin in circadian rhythm generation, seasonal cycles and sleep has been extensively described (Brown, 1994; Morgan and Mercer, 1994). Because serotonin synthesis is regulated by modulating the rate of conversion of 1-tryptophan to 5-hydroxytryptophan (Bodale-Biber, 1993), there is evidence that \(BH_4\) participates in the short-term control of 5-hydroxytryptophan synthesis. Controlling serotonin levels in the appropriate regions of the brain has an obvious therapeutic benefit because of multiple effects of this compound with regard to tranquilizers, hallucinogens and antidepressants. In contrast to tyrosine hydroxylase,
tryptophan hydroxylase is not subject to inhibition by the end product of the reaction pathway (Joh et al., 1975). Since in the brain the level of the pteridine co-factor is relatively low and may be limiting, rapid modulation of tryptophan monoxygenase activity could occur in vivo by small changes of an available pool of BH4 to control the regulation of the biosynthesis of serotonin in brain (Kaufman, 1975). The specific and strong inhibitory complex between SR and NAS gives evidence for a feedback regulatory mechanism between the formation of BH4 and indoleamines in vivo. Additionally, the complex with noradrenaline raises the possibility of a similar mechanism controlling the biosynthesis of catecholamines at the reaction step of SR.

Furthermore, the exceptionally high resolution of the crystal structure of SR may facilitate rational drug design also for other single-domain oxidoreductases, such as the HSDHs, implicated in controlling blood pressure or in breast cancer, or the non-SDR oxidoreductase InhA, a drug-target of Mycobacterium tuberculosis for AIDS patients, who develop tuberculosis.

Materials and methods

Cloning
The native cDNA sequence coding for the murine SR was adapted to the codon usage of highly expressed proteins of E. coli (Grantham et al., 1981) and cloned in the expression vector p602-CAT (Mört et al., 1996), yielding the plasmid p602-SRP13. The E. coli M13spGB3 host strain (Stiher et al., 1999), carrying the pGB3 repressor plasmid for the overexpression of the lac repressor protein, was transformed with the plasmid. In this strain the cDNA is under control of the T5 promoter and the lac operator.

Overexpression and purification
A culture of recombinant E. coli was grown in Terrific Broth medium (Sanbrook et al., 1989) containing 150 mg/l ampicillin and 20 mg/l kanamycin. In the late logarithmic phase the culture was induced by addition of isopropyl-β-D-galactoside to a final concentration of 2 mM and was harvested at 18-24 h after induction. The cells were centrifuged (5000 r.p.m., 10 min, 4°C), washed with 0.9% NaCl, re-centrifuged and resuspended in 5 ml of buffer A (10 mM MES, 2.5 mM EDTA, 1 mM DTT, 0.02% NaN3, pH 6.5) per 1 g of wet cells. Bacteria were lysed by ultrasonic treatment, and the cellular debris was removed by centrifugation (15 000 r.p.m., 20 min, 4°C). The supernatant was loaded on a Matrix Red A Sepharose column equilibrated with buffer A. The column was washed successively with 4 vols of buffer A containing 0.5 mM NADH and 3 vols of buffer A. The protein was eluted with 1 vol. of buffer A containing 0.5 mM NADP and 1 vol. of buffer A. The enzymatic activity of SR was monitored as described previously (Ferre and Naylor, 1988; Kerler et al., 1990). Fractions were constantly cooled to 0°C. The pH of the solution was adjusted to pH 8 by the addition of 10 mM NaOH and kept constant during the slow addition of solid (NH4)2SO4 to a final concentration of 1.5 M. After 30 min the precipitated protein was harvested by centrifugation (15 000 r.p.m., 20 min, 4°C). The supernatant was applied to a Butyl-Sepharose column equilibrated with buffer B (10 mM MES, 2.5 mM EDTA, 0.02% NaN3, 1 mM DTT, 1.5 M (NH4)2SO4, pH 6.0). After washing the column with 2 vols of buffer B, the protein was eluted with a linear gradient [1.5-0 M (NH4)2SO4] over 15 vols. Fractions were monitored by enzyme assay and SDS–PAGE. Fractions showing a single protein band were combined and dialysed against 10 mM MES, pH 6.5. The solution was centrifuged for 20 h at 55 000 r.p.m., 4°C. The supernatant was removed and the colourless protein pellet was dissolved overnight at 4°C.

Crystalization
Crystallization experiments were performed at 4°C employing the vapour diffusion technique. Crystals from nSR protein (5.5 mg/ml protein
concentration), buffered in 20 mM MES, pH 6.5, 1 mM DTT, 10 mM NaN₃ appeared within 12 days using 1.5 mM ammonium sulfate, buffered with 0.1 M citrate to pH 4.7, and grew within 3 weeks to a size of 400 μmx600 μm. The crystals are hexagonal bipyramids and belong to the space group P6₃22 with lattice constants of a = b = 116.8 Å, c = 104.78 Å, γ = 120°. The asymmetric unit contains one molecule SR as determined by volume measurement and quantitative amino acid analysis of three crystals of different volume (Kiefersauer et al., 1996). The crystals have a solvent content of 66% (V₅ₐ₅ = 3.66 Å³/DA) and diffract to a resolution of 1.2 Å. The crystal packing is honeycomb-like with the walls formed by protein. The hexagonal c-axis is centered in each comb with a diameter of 75 Å, which explains the significant anisotropy of the temperature factors especially in the direction of the c-axis, as described later.

Data collection and soaking conditions

Data sets to 1.9 Å resolution were collected with a Mar Research imaging plate system installed on a Rigaku rotating anode generator by taking 1° frames for at least 30° around the c-axis. Native diffraction data to a resolution of 1.22 Å were collected from a cryo-cooled crystal at the BW6 beamline at DESY (Hamburg) (λ = 1.0 Å, 0.5° frames). Under cryo conditions the cell constants were reduced to a = b = 115.8 Å and c = 103.78 Å. The crystals were mounted into a fibre loop, which was prepared in a pulled-out microirrigation capillary. The glass capillary was fixed into a magnetic basis for fast mounting on a goniometer head. The crystals were flash-cooled in a 95 K nitrogen beam and the crystals were cryoprotected. Data from heavy atom derivatives were obtained at 16°C by soaking crystals at 4°C either for 3 h in a solution containing 1 mM Hg(OAc)₂, for 1 day in 5 mM TaBr₄, or for 3 h in 1 mM UO₂(NO₃)₂. The complex structure with the substrate sepiapterin was obtained by soaking crystals for 30 min in a solution containing 10 mM sepiapterin. The crystals coloured yellow during soaking with sepiapterin and maintained this colour during the entire data collection period. The complex with the product B₈L₂ was prepared after 15 min preincubation of the crystals with reduced NADPH by soaking for 30 min with a solution of 10 mM B₈L₂. The enzyme was complexed with NAS by soaking crystals for 30 min with a solution of 5 mM NAS.

A reproducibly expressed protein already contained oxaloacetate bound to the active site. Soaking crystals of oxaloacetate-containing protein with NAS led to the formation of a quaternary complex of SR with NAS, OAA and NADP. In order to analyse the crystal structure of a ternary complex of the enzyme with NAS, but without OAA, the enzyme was incubated overnight with 100 μM sepiapterin for replacing OAA from the active site, and then treated with charcoal for removing the sepiapterin. The protein was crystallized and soaked under the same conditions as mentioned above. The same protein, treated with sepiapterin and charcoal, was used for co-crystallization with 10 mM sodium malonate.

Data processing and phasing

All data were evaluated with MOSFLM (Leslie, 1991) and CCP4 (1994). A total of 112 800 unique reflections (630 228 total measurements) was collected for the native data set, representing 95.6% of the possible data to a resolution of 1.25 Å. The data are 99.9% complete to a 1.28 Å resolution and 97.3% in the 1.28-1.25 Å shell. The Rmerge on intensities is 6.8%. Initially, we tried to solve the structure of mSR by molecular replacement techniques. The structure of 3t2b:HSDH (Ghosh et al., 1994) with a sequence identity of 24% was used as a starting model. Because this attempt failed, multiple isomorphous replacement was used for phase determination by locating heavy atom sites from isomorphous difference Patterson functions and confirming them by cross-difference Fourier syntheses. MIR phases to 2.5 Å from three derivatives [Hg(OAc)₂, TaBr₄, UO₂(NO₃)₂] were refined using the programs PROTEIN (Steigemann, 1974) and MLPHARE (CCP4, 1994) resulting in an overall figure of merit of 0.64 (20-3.0 Å) (Table 1). The search group P6₃22 was distinguished from its enantiomorph P6₃22 with a consistent set of heavy atom sites after inclusion of anomalous dispersion data.

Model building and refinement

Model building was done with FRODO and O (Jones et al., 1990) and refinement with X-PLOR (Leng and Huber, 1991; Brünger, 1992). After solvent flattening with DM (Cowtan and Main, 1996) almost the complete model could be built into the density. The initial model built gave an R-factor of 40.5% (Rmerge = 46.8%) after refinement with X-PLOR using the native data set NAT2. After one round of model building and combination of model and experimental phases using SIGMAA (Read, 1986), the complete model comprising 261 amino acids had been placed in the density and the R-factor had decreased after refinement to 24.7 Å (Rmerge = 28.4%, data from 8.0 to 2.0 Å). No solvent molecules have been placed into the 2.0 Å electron density (NAT2). By increasing the resolution stepwise, the model was refined to 1.25 Å using the native data set NAT1. At a resolution of 1.4 Å and an R-factor of 24.7% (Rmerge = 27.7%) all polar hydrogens were included and the Fobs were scaled to the Fcalc by using overall anisotropic B-factor refinement by means of X-PLOR resulting in a direction-dependent correction for the vibration ellipsoid of -2.36 Å along the axes α* and β* (B₁₁, B₂₂) and -5.66 Å along c* axis (B₃₃). The current model, refined to 1.25 Å, comprises 2346 C, N, O atoms, and 1410 hydrogen atoms. 484 solvent molecules have been placed solely into a well-defined Fobs-Fcalc electron density contoured at 3 σ and in a 2Fobs-Fcalc electron density contoured at 10 σ. Two ions of sulfate are bound to the residues Arg220 and Arg79, the latter in close proximity to the co-factor-binding residue, Arg43.

Finally, alternative conformations were refined for six side chains. The crystallographic R-factor of the model is 20.0% for all unique reflections from 8.0-1.25 Å resolution (Rmerge = 22.2%). The r.m.s. deviations from ideal stereochemistry are 0.007 Å for bond lengths and 1.58° for bond angles (Engl and Huber, 1991). The stereochemistry of the model was verified using the program PROCHECK (Laskowski et al., 1993). The dihedral angles of the polypeptide backbone all lie in allowed regions of the Ramachandran diagram. The model refined to 1.25 Å resolution was used as an apo model for refinement of all other crystal structures after rigid body refinement by means of X-PLOR. The ligands were built into the resulting difference electron density and were included for final refinement. Ribbons representations of the main-chain folding of the molecule were drawn using the programs MOLSCRIPT (Kraulis, 1991) and RENDERR (Merritt and Murphy, 1994). The coordinates have been deposited with the Protein Data Bank Brookhaven, NY, under accession numbers 1SEP and IOAA.

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