Bile salt hydrolase (BSH) is an enzyme produced by the intestinal microflora that catalyzes the deconjugation of glycine- or taurine-linked bile salts. The crystal structure of BSH reported here from *Bifidobacterium longum* reveals that it is a member of N-terminal nucleophil hydrolase superfamily possessing the characteristic αββα tetra-lamellar tertiary structure arrangement. Site-directed mutagenesis of the catalytic nucleophil residue, however, shows that it has no role in zymogen processing into its corresponding active form. Substrate specificity was studied using Michaelis-Menten and inhibition kinetics and fluorescence spectroscopy. These data were compared with the specificity profile of BSH from *Clostridium perfringens* and penicillin V acylase from *Bacillus sphaericus*, for both of which the three-dimensional structures are available. Comparative analysis shows a gradation in activity toward common substrates, throwing light on a possible common route toward the evolution of penicillin V acylase and BSH.

Bile salt hydrolase (BSH) (cholylglycine hydrolase; EC 3.5.1.24) catalyzes hydrolysis of the amide bond in conjugated bile salts, resulting in the release of free amino acids. Bile salts are synthesized mainly from cholesterol by conjugation with the amino acids glycine or taurine in the liver, and stored in the gall bladder until their release into the duodenum in response to ingestion of fatty foods (1, 2). Bile salts are also the natural ligands for the farnesoid-X nuclear receptor. Therefore, they are considered important regulators of gene expression in the liver and intestines (3). Upon completion of emulsification, bile acids are returned to the liver by an active transport mechanism. However, their hydrolysis by the bacterial enzyme BSH results in production of free bile acids, whose affinity for the transport system is diminished (4, 5). These bile acids are then passed into the large intestine where they are further metabolized.

As conjugated bile salts possess antimicrobial activity, bacteria seem to have evolved to produce BSH to neutralize this adverse activity (6, 7). A number of bacterial strains possessing de-conjugating activity such as *Enterococci, Bacteroides*, anaerobic *Lactobacillaceae*, and *Clostridia* have been isolated (8–12) and these have been shown to be present in ileal and fecal content. *Bifidobacteria* are among the most common genera in the human colon and have been considered as key commensals in promoting host health, but very little is known about their genetics (13). *Bifidobacterium longum* is the most studied among the 32 species of *Bifidobacteria* known today (14).

Depleted levels of bile salts following their hydrolysis triggers consumption of cholesterol, resulting in further synthesis of bile salts with a consequential lowering of the serum cholesterol levels. Thus, in addition to an effective increase in bile tolerance levels, enhancing the BSH activity of probiotics offers a potential biological alternative to pharmaceutical interventions for treating hypercholesterolemia (15–17). Possible detrimental effects due to the introduction of BSH activity have also been hypothesized. De-conjugated bile salts are implicated in the formation of gallstones (18), in the retarded growth of chickens due to poor lipid uptake by the small intestine (19), and in colorectal cancer (20). Furthermore, it has been proposed that BSH activity in virulent strains of *Listeria monocytogenes* contributes to virulence (21). The clinical significance of BSH warrants structural and biochemical studies that can reveal the determinants of its activity modulation.

The conspicuous sequence similarity of 29% between BSH and penicillin V acylase (PVA) suggested that the enzyme would belong to the N-terminal nucleophil (Ntn) hydrolase superfamily. Although the Ntn hydrolases display a wide range of substrate specificity, the self-activation and catalytic mechanisms of these enzymes seem similar. All the known members of the family catalyze the hydrolysis of amide bonds present in proteins or in small molecules, and each one of the members is synthesized as a pre-protein. An autocatalytic endoproteolytic
process is thought to generate a new N-terminal residue, which is designed to act as a nucleophile. For both BSH and PVA, Cys is predicted to be the first residue of the mature protein. This residue is central to the mechanism of catalysis and serves both as a nucleophile and as a proton donor (22). The N-terminal amino group acts as the proton acceptor and activates the nucleophilic thiol group of the Cys side chain. Cys1 becomes a catalytic center only on removal of the initiation formylmethionine. Such unmasking post-translational modifications are common to all members of the Ntn hydrolase superfamily (23). The importance of the -SH group was confirmed by the fact that replacement of Cys with other potential nucleophilic residues such as Ser or Thr resulted in the loss of BSH activity (24).

Sequence analysis of BSH suggests that its tertiary structure, specifically the special arrangement of catalytic residues, might be similar to that of PVA. This prompted us to test the action of BSH on penicillin V (penV, Fig. 1) as well as other related compounds like penicillin G (penG), and also to test the inhibitory effects of compounds such as phenylacetic acid (PAA) on the enzyme activity. This is expected to provide insight into the construction of the catalytic site of BSH. Classical inhibition kinetic studies demonstrate the type of inhibition and its quantitative aspects. Furthermore, the active site protection assay suggests that the inhibition is due to the binding of inhibitor at, or near, the active site itself. Transient structural changes during the binding of inhibitors were monitored through fluorescence microscopy. Tryptophan proved to be a useful intrinsic probe, because its fluorescence emission spectrum varied depending on the molecular environment. Disruption of the native structure leads to changes in the exposure of the tryptophan side chains to solvent that can be readily monitored by recording the protein fluorescence emission spectrum. Here we report the crystal structure of BSH from B. longum and comparative studies of structure-function between PAVs and BSHs that suggests a common catalytic mechanism and possible evolutionary relationship between these two enzymes.

**EXPERIMENTAL PROCEDURES**

**Materials**—Conjugated bile salt kit, penicillin V, penicillin G, PAA, phenoxycetic acid (POAA), 4-amino PAA, cholic acid, deoxycholic acid, ninyhydrin, diethiothreitol, and Sephacryl S-200 were obtained from Sigma. All chemicals used were of analytical grade.

**Overexpression and Site-directed Mutagenesis**—The B. longum bsh gene was amplified from plasmid pBH1351 (25) by PCR, using primers GGAGTCATAATTGTGACTGTTGCCGTTC (A) and GGAAGATTACATCGGCGACGCTGTATGA (B), which incorporate restriction enzyme recognition sites. C1A and T2A mutations were introduced by appropriate substitutions in primer A, (GGAGTCATAATTGCCACACTGGTGCCGTTTC and GGAGTCATTATGTGCGCTGTGTCCGGTTTT, respectively). The PCR product was cloned into the T7 promoter-based pET26b(+) expression vector (Novagen) using Ndel and EcoRI restriction sites and transformed into Escherichia coli BL21(DE3) for protein production.

**BSH Protein Purification and Crystallization**—BSH protein was purified and crystallized essentially as described earlier (26). Crystals of the two mutants were obtained in similar conditions with slight differences in the concentration of additives. Clostridium perfringens BSH was obtained from Sigma and purified by passing through Sephacryl S-200. Recombinant penicillin acylase from Bacillus sphaericus was purified as described previously (24).

**Structure Determination and Refinement**—The structure was solved by molecular replacement using one molecule of PVA (3PVA) as a search model (the structure 2BF reported by Rossocha et al. (27) is a closer match but it was not available at the time). Two copies were positioned using the program MOLREP (28) within the CCP4 crystallographic software suite (29). The solution in space group P32_1 was clearly better than in the enantiomorph, P32_2. As expected the two molecules form a dimer in the asymmetric unit, and the complete homotetrameric unit is generated by the crystallographic 2-fold axis. The model was completed using automated model building program ARP/wARP (30) and refined using the REFMAC program (31). This resulted in an initial model with an R-factor of 27.0 and Rfree of 31.0. Subsequent rounds of refinement were carried out alternating between manual model building with COOT (32) and refinement using the maximum likelihood method. In the last four refinement cycles, solvent molecules were placed at peaks of (Fo − Fc) density above 4σ (3σ in the final cycle) providing the sites could participate in hydrogen bonds with protein atoms. 229 water molecules were fitted into the asymmetric unit. The difference map (Fo − Fc) showed three distinct blobs of density close to the Sβ atom of N-terminal residue Cys1. These were interpreted as oxygen atoms due to formation of sulfonic acid. Extra density in the vicinity of the N-terminal cysteine was left unidentified and may represent an oxidized diethiothreitol molecule. Structure validation was performed using the program PROCHECK (33), which showed all 316 residues in the allowed region of a Ramachandran map (34). The structural homology search using the three-dimensional structure against the data base of protein chains was carried out using the program DALI (35). The structures of C1A and T2A mutants of BSH were determined using the refined model of wild-type BSH as a search model in MOLREP. Other steps were carried out as described.

**Bile Salt Hydrolase Assay**—BSH enzyme activity was determined by estimating the amount of free amino acids released upon incubation of the enzyme sample with 1 mm sodium taurocholate or 1 mm sodium glycocholate at 40°C in 10 mm sodium phosphate, pH 6.5, containing 10 mm diethiothreitol. After 10 and 30 min incubation time, a 25-μl aliquot was with-
Structure of Bile Salt Hydrolase from B. longum

drawn and the reaction arrested by mixing with 25 μl of 15% (w/v) trichloroacetic acid. The sample was spun at 10,000 × g for 1 min and the supernatant was mixed with an equal volume of 2% ninhydrin solution before boiling for 15 min. The absorption was recorded at 570 nm and the amount of product formed was estimated from a calibration curve (36). One unit of BSH activity is defined as the amount of enzyme that liberates 1 μmol of the amino acid from substrate per min. Specific activity was defined as the number of units of activity per milligram of the pure protein.

Substrate Specificity—The values of \( K_m \) and \( k_{cat} \) for different substrates were determined by incubating the enzyme sample with a range of substrate concentrations under standard assay conditions. Non-substrate ligands were compared by determining the \( K_i \) measured by incubating 1.12 mg/ml of BSH with the respective inhibitors in the concentration range 0.5–5 mm and by increasing the concentration of glycocholic acid (GCA; 0.1–10 mm) under standard assay conditions. The constants were calculated by fitting the linear regression curve to the data on Lineweaver-Burk plots using Enzyme Kinetics!Pro (37). The standard errors in the estimation of the values of \( K_m \) and \( k_{cat} \) were within limits of 10%.

Cysteine Modification Active Site Protection Assay—BSH (4 μM, 1 ml) in 100 mM potassium phosphate buffer, pH 8.0, was incubated with 1 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) at 30 °C for 60 min. Aliquots were removed at defined time intervals and the residual activity was determined. The protective effect of substrate and inhibitor during cysteine modification was determined by incubating the enzyme with an excess of substrate or inhibitors prior to treatment with modifying reagent under similar experimental conditions. Excess reagents were removed by passing through a PD10 column. The residual activity was quantified under the standard assay conditions.

Fluorescence Measurement—Fluorescence was measured using a PerkinElmer Life Sciences LS50 fluorescence spectrophotometer connected to a Julabo F20 water bath. To eliminate background emission, the signal produced by either buffer solution, or buffer containing the appropriate quantity of substrate or inhibitor was subtracted. The sample was excited at 295 nm and the emission recorded in the 300–400 nm wavelength range at 25 °C. The slit-width on both the excitation and emission were set at 5 nm, and the spectra were obtained at 100 nm/min. The binding constant \( (K_d) \) of the various substrates and inhibitors was determined by monitoring the effect of fluorescence emission upon titration with a given ligand (38–39). The binding of ligands to BSH can be described as,

\[
K_d = \frac{[\text{BSH}] [\text{inhibitor}]}{[\text{BSH inhibitor}]} \quad \text{(Eq. 1)}
\]

where \( K_d \) is the apparent dissociation constant, [BSH] is the concentration of the protein, [BSH inhibitor] is the concentration of complexed protein, and [inhibitor] is the concentration of unbound inhibitor. The proportion of inhibitor-bound protein as described by Equation 1 is related to measured fluorescence emission intensity as,

\[
(F_o - F) / (F - F_o) = [\text{BSH inhibitor}]/[\text{BSH}] \quad \text{(Eq. 2)}
\]

where \( F_o \) is fluorescence intensity of enzyme alone, \( F \) is observed fluorescence intensity at a given concentration of inhibitor, \( F_o \) is intensity of BSH saturated with inhibitor, and \( [\text{BSH}] \) is the total protein concentration. If the total inhibitor concentration, [inhibitor] \( T \), is in large molar excess relative to [BSH] \( T \), then it can be assumed that [inhibitor] is approximately equal to [inhibitor] \( T \). Equations 1 and 2 can then be combined.

\[
(F_o - F) / (F - F_o) = [\text{inhibitor}] / ([K_d] + [\text{inhibitor}]) \quad \text{(Eq. 3)}
\]

The values of \( K_d \) were determined from a nonlinear least square regression analysis of titration data using Equation 3. The stoichiometry of binding was established using a linear version of the Hill equation,

\[
\log((F_o - F)/(F - F_o)) = n\log[\text{inhibitor}] - \log K' \quad \text{(Eq. 4)}
\]

where \( n \) is the order of the binding reaction with respect to inhibitor concentration and \( K' \) is the concentration of the ion that yields 50% of \( F - F_o \). The thermodynamic parameters \( \Delta G \), \( \Delta H \), and \( \Delta S \) were determined according to Equation 5,

\[
-RT \ln K = \Delta G = \Delta H - T \Delta S \quad \text{(Eq. 5)}
\]

where \( R \) is the gas constant, and \( T \) corresponds to absolute temperature.

RESULTS

The crystal structure of wild-type BSH has been determined at 2.5-Å resolution (Table 1) and its mutants C1A and T2A at 3.2 and 3.0 Å, respectively (details not included). The wild-type protein crystallized in space group \( P3_12_1 \) with unit cell param-
eters \(a = b = 125.24, c = 117.03\) Å and in P6\(_1\)2 space group with cell parameters \(a = b = 123.98, c = 219.56\) Å (Table 1). One of the mutants, C1A, crystallized in space group C2 with unit cell parameters \(a = 186.36, b = 71.10, c = 133.65\) Å, and \(\beta = 109.10^\circ\), whereas the other, T2A, crystallized in the same space group and cell parameters as the wild type (data not shown). Reflections were phased using the molecular replacement method. The processed form of the \(B.\ sphaericus\) penicillin V acylase monomer (3pva) was used as a search model. The initial electron density allowed assignment of all 316 residues in each of subunits A and B of the asymmetric unit. The overall G factor output by PROCHECK, considered as a measure of stereochemical quality of the model, is \(-0.034\). This is within the limits expected for a structure that is refined at 2.5-Å resolution. The refinement statistics are presented in Table 1.

Description of the Structure—The overall structure of BSH from \(B.\ longum\) (BlBSH) confirms the characteristic Ntn hydrolase fold comprised of a four-layered \(\alpha\beta\alpha\beta\) core structure that is formed by two anti-parallel \(\beta\)-sheets packed against each other, with these \(\beta\)-sheets sandwiched between the layers of \(\alpha\)-helices (Fig. 2a). The approximate monomer dimensions are 75 \(\times\) 38 \(\times\) 44 Å. \(\beta\)-Sheet I includes the N and C termini and is composed of six strands. \(\beta\)-Sheet II comprises seven strands. The topology of the strands in the first \(\beta\)-sheet is \(\text{NH}_2\beta_1\beta_2\beta_3\beta_4\beta_5\beta_6\beta_7\) and the second \(\beta\)-sheet is \(\beta_1\beta_2\beta_3\beta_4\beta_5\beta_6\beta_7\beta_8\beta_9\beta_10\beta_11\) (Fig. 2b). \(\beta\)-Sheet I is more flattened and the angle between the strands of the two sheets is 30°.

Few residues are involved in the loops connecting the \(\beta\)-strands and \(\alpha\)-helices. However, a large loop of about 26 Å length comprising residues 188 to 220 contained in \(\beta\)-sheet II is a prominent feature in the structure, which extends into the neighboring molecule of the tetramer. Two other major loops (58–65 and 129–139) enclose the active site. The distances from active site residue Cys\(^1\) to the termini of the above two loops are about 15.5 and 13.4 Å, respectively. Of a total of 12 loops, nine contain glycine residues that are more flexible in their possible conformation than other amino acids and influence the loop structure. These glycine residues appear to be conserved in all the Ntn hydrolases. There is a preponderance of hydrophobic residues between the \(\beta\)-sheets and many hydrophobic side
chains are also present in the interface between β-sheets and α-helices and between α-helices. The β-sheet II and α-helix I layer together form a substructure reminiscent of a T-fold.

**Tetrameric Structure**—The tetrameric association of BSH subunits in solution was suggested by gel filtration and dynamic light-scattering studies (data not shown). The tetramer may be considered a dimer of dimers. In the structure, the dimer forms a tetramer with its replicate monomers, referred to as AS and BS generated by a crystallographic dyad axis (Fig. 3). A major contribution to the formation of the dimer in the asymmetric unit comes from interaction of the loop consisting of residues 188–200. The buried surface area between subunits A and BS is about 10%. About 26% of the total tetramer surface is involved in subunit interaction. Thus the maximum surface is 43,500 Å².

In the tetramer, each monomer interacts with the remaining three monomers. The buried surface area between monomers A and AS is 13% of the surface of each monomer. The main interactions between these monomers are made by the long loop (residues 188–200). The buried surface between subunits A and B is only 3% of the monomer surface area, whereas that between A and BS is about 10%. About 26% of the total tetramer surface is involved in subunit interaction. Thus the maximum interactions within the dimers are between subunits A and AS or B and BS. The dimers are held together by the interaction of A with B and AS with BS. Each of the monomers has approximately one-fourth of the surface contributed in tetramer formation.

**Active Site and Activity Studies**—Chemical modification of BSH with 2 mM DTNB lowered the activity by 78%. In the presence of 20 mM GCA, the drop in activity was only 12% indicating that the modified residue or residues are at, or near the active site. The conformity between the CD spectra of the wild type and the chemically modified BSH indicates that the loss of activity is due to specific residue modification and not due to structural perturbations. The putative role of Cys¹ in catalysis was further clarified by the replacement of Cys¹ by Ala through site-directed mutagenesis. The C1A mutant turned out to be completely inactive. The residue Cys¹ is situated at the tip of strand β1. The proximity of this residue to other putatively important residues such as Trp²¹, Thr¹⁷¹, Asn¹⁷², and Arg²²⁵ is evident from the structure (Fig. 4a).

The binding site was also confirmed by superposing the structure of the enzyme-substrate complex of BSH from *C. perfringens* (CpBSH) (27). The residues that are identified as responsible for catalysis (Cys¹, Arg¹⁷, Asp²⁰, Asn¹⁷⁴, and Arg²²⁷) by comparison with penicillin V acylase (41) are conserved in both the structures (Fig. 5). Residues involved in substrate binding are conservatively replaced. The substrate-binding site consists of residues of β-sheet II located in strands β₄, β₅, and β₉ that stabilize the geometry of the active site. The higher B-factors of the loop comprising residues 262–273 that are proximal to the active site reveal its enhanced conformational flexibility (Fig. 4b). Based on sequence alignment (Fig. S1) and substrate binding studies we suggest that Trp²¹ plays a selective role in binding of bile salt while it suppresses productive binding of penV. This residue is located about 6.3 Å away from Cys¹.

**Crystal Structures of C1A and T2A**—The superposition of the mutant and wild-type BSH structures showed that there was little effect of the mutations on the geometry of the catalytic site. Residue Thr² is conserved in all BSHs but not in PVA where serine takes its place (Fig. S1). We decided to test this mutation for possible effects on processing or activity. Both mutant proteins process normally, by simple removal of the initiator methionine. The C1A mutant is completely inactive, whereas T2A mutant is partially active, with a reduced $k_{cat}$ (Table 2).

**Substrate Specificity**—BBBSH exhibited a preference for glycine-conjugated bile salts over taurine-conjugated forms, but did not appear to discriminate between deoxy- and dihydroxy-bile salts. BSH activity of enzymes CpBSH and BsPVA are 73 and 20%, respectively, of the BBBSH activity. The latter is totally inactive toward penicillin V, whereas the BsPVA enzyme has the highest PVA activity. The penicillin V acylase activity of CpBSH is only 11% of the activity of BsPVA. There appears therefore to be an inversion of substrate preference within this protein set. For comparison, the values of their activities at their respective pH optima have been tabulated (Table 3).

**Inhibition Kinetics**—Reaction products and substrate analogs were tested for their inhibitory effects. Initial kinetic assessments revealed that CA and DCA competitively inhibited bile salt hydrolase. The other related compounds like POAA, PAA, and 4-amino-PAA also showed competitive inhibition, whereas penV and penG showed mixed inhibition. The $K_i$ values of these compounds are listed in Table 4.
In an independent method to determine the value of $K$ (the rate constant for the conversion of conjugated bile salt to deconjugated bile salt), high concentrations of enzyme and inhibitor were preincubated for sufficient time to allow the system to reach equilibrium. The enzyme-inhibitor mixture was then diluted 100-fold and assayed at saturated substrate concentration ($100K_m$), which resulted in the dissociation of inhibitor and regeneration of enzyme activity.

The inhibition of enzymatic activity by penV was investigated, given that this is a substrate mimic of conjugated bile acid. From Lineweaver-Burk plots, penV shows mixed inhibition against the hydrolysis of GCA. To characterize the system, both Dixon (i.e. $1/V$ versus $I$) and Cornish-Bowden (i.e. $S/V$ versus $I$) plots were constructed (not shown). On a Dixon plot the five regression lines corresponding to various GCA concentrations intersected at a point that was clearly negative for both $1/V$ and $I$.

It is interesting to note that a mixed form of inhibition has been observed in the case of penV and penG, whereas it is strictly competitive for CA and DCA. This implies that the conformation of the enzyme active site in complex with inhibitor may be substantially different from that formed with CA and DCA. The pattern shown by the kinetic results can be understood in terms of complementarity between the structure of the inhibitor and the binding site. Evidently, a more elaborate study with enzyme-substrate complex will be required to record the diverse interactions between enzyme and its substrates or inhibitors such as penV.

**Active Site Protection**—The affinity of various ligands for BSH enzyme was determined by their protective effect during modification of the active site cysteine by DTNB. The degree of protection offered by various ligands is tabulated in Table 5 as percentage of residual activity with reference to the activity of unmodified sample.

**Fluorescence Measurement**—The fluorescence spectra of BSH showed emission maxima at 333.5 nm ($\lambda_{max}$). The 315-amino acid sequence of the protein has five tryptophan and 12 tyrosine residues. To excite Trp selectively, a wavelength of 295 nm was used. The titration with substrates (GCA and GDCA) as well as with the end products (CA and DCA) both resulted in enhancement of fluorescence. Other ligands tested quenched the fluorescence. The absorption spectra of ligands do not overlap with the tryptophan fluorescence spectrum; hence, an
energy transfer from tryptophan to ligands may be ineffectual. To rule out the possibility of collision quenching of tryptophan fluorescence, we measured the fluorescence parameters of free tryptophan in the presence of all ligands. Fluorescence of free tryptophan is not quenched by the ligands; thus, the collision mechanism of BSH fluorescence quenching is ruled out. Fig. 6a shows the quenching of tryptophan fluorescence as a result of penicillin V binding to BSH. When denatured BSH was used, no quenching of the intensity due to tryptophan fluorescence occurred in the presence of penicillin V. Therefore it can be concluded that the quenching of tryptophan fluorescence of the active form of BSH is a direct consequence of enzyme-substrate/inhibitor interaction.

From the changes in fluorescence spectra, the dissociation constant and enthalpy change for ligand binding were calculated for penV (4.6 × 10⁻⁹ and −61.1 kJ/mol) and penG (5.2 × 10⁻⁴ and −62.7 kJ/mol) and found to have similar favorable values. The slope of the plot of \( \log(F_o - F)/(F - F_o) \) versus \( \log[S] \) was unity for all the inhibitors used, indicating the formation of a 1:1 complex between enzyme and inhibitor (Fig. 6b). The titration with penicillin G resulted in a red shift of the fluorescence maxima from 333.5 to 338 nm (not shown).
DISCUSSION

Structural Comparison of BSH with PVA—The structure of BBSH is highly similar to those of CpBSH (27) and BsPVA (41). On superposition of 294 and 291 equivalent BBSH Ca atoms, the root mean square deviation between CpBSH and BsPVA is 1.9 and 2.1 Å, respectively (Fig. 7). The pattern of secondary structures is also similar in all three proteins. The β-sheet arrangements are shown in Fig. 2b. BBSH contains an additional β-strand in the first sheet. The prominent difference observed between these three structures is the reduced length of the C-terminal tail in the case of BBSH. This extra strand, present in CpBSH and BsPVA provides additional subunit interaction by forming an intermolecular β-sheet. So the association of the subunits in BBSH might not be as strong as in CpBSH and BsPVA due to the absence of these C-terminal interactions. Further investigation is required to understand the significance of this terminal strand in subunit interaction.

Detailed examination of the superposed structures of these three enzymes has shown that there is significant difference in the orientation of the loops near the active site. Interestingly in CpBSH, only these loops are displaced in the ligand bound enzyme compared with free enzyme. There is considerable movement of loops comprising residues 60–65 and 125–144 and minor changes in loops of residues 35–50 and 20–27. The largest displacement of 10 Å is observed between loop region 60–65 of BsPVA and of BBSH. All of the above loops might be playing a role in substrate specificity. These loops are more open and placed further away from the binding pocket as we go from the structure of a "good" PVA to a "good" BSH (Fig. 4b). In

![Table 4](image)

**Table 4**

Inhibition constants (mean ± S.D.) for bile salt analogs binding to bile salt hydrolase

| Compounds               | Inhibition type | $K_m$ or $K_i$ |
|-------------------------|-----------------|----------------|
| GCA                     | 0.22 ± 0.07     |                |
| Glycodeoxy cholic acid (GDCA) | 0.18 ± 0.05     |                |
| Glycochenodeoxy cholic acid (GCDC) | 0.28 ± 0.11     |                |
| Taurocholic acid (TCA)   | 0.32 ± 0.13     |                |
| Taurodeoxycholic acid (TDCA) | 0.49 ± 0.04     |                |
| Taurochenodeoxy cholic acid (TCDC) | 0.42 ± 0.15     |                |
| CA                      | Competitive     | 0.32 ± 0.07    |
| DCA                     | Competitive     | 0.46 ± 0.09    |
| PAA                     | Competitive     | 0.94 ± 0.17    |
| POAA                    | Competitive     | 0.82 ± 0.21    |
| Phenoxy penicillin      | Mixed           | 1.20 ± 0.32    |
| Benzyl penicillin       | Mixed           | 2.00 ± 0.48    |
| 4-Amino phenyl acetic acid | Competitive    | 0.86 ± 0.26    |

$K_m$ is Michaelis-Menten constant and $K_i$ is inhibition constant ($K_{app} = K_m [1 + I/K_m]$, where $K_{app}$ is the apparent $K_m$ in the presence of inhibitor $I$, concentration of inhibitor).

![Table 5](image)

**Table 5**

Active site protection provided by various ligands during cysteine modification by DTNB (20 mM)

| Treatment                | Activity % |
|--------------------------|------------|
| Enzyme alone             | 100        |
| DTNB                     | 25 ± 1     |
| CA + DTNB                | 75 ± 4     |
| DCA + DTNB               | 72 ± 2     |
| PAA + DTNB               | 84 ± 6     |
| POAA + DTNB              | 50 ± 4     |
| 4-amino PAA + DTNB       | 62 ± 2     |
| PenV + DTNB              | 75 ± 6     |
| PenG + DTNB              | 74 ± 4     |

![Figure 6](image)

**Figure 6.** a, fluorescence quenching observed due to penicillin V binding obtained at different concentrations: no added substrate (solid), 0.5 (—), 0.75 (●), 1 (◆), 1.5 (□), and 2 mM (●). b, plot of log ($K_m$ ($F_o / F$) / ($F/F_o$)) against log [Molarity] of penicillin V.

![Figure 7](image)

**Figure 7.** The root mean square deviation ($y$ axis, in Å) estimated between the positions of main chain atoms of (a) BBSH versus BsPVA and (b) BBSH versus CpBSH. The arrows indicate loop regions identified as involved in substrate binding or specificity and those that show comparatively large deviation.
Structure of Bile Salt Hydrolase from B. longum

BsPVA it is nearer to a closed conformation, whereas it has a more open conformation in BBSH. In general the surface loops show considerable deviation between the three structures.

Comparison with Other Ntn Hydrolases—A number of crystal structures of the protein from the N-terminal nucleophile hydrolase family have been solved (Table S1). Many amidohydrolases, including penicillin acylase, glutamine PRPP amido- transferase, the proteasome, and glucosamine-6-phosphate synthase have the αββα architecture as a protein framework.

Even though their primary sequences share very limited homology and a wide range of variation in their quaternary structure association exists, the members of the Ntn hydrolase family have a well conserved arrangement of critical residues and an equivalent stereochemistry is preserved at the active site, even when the nucleophilic residue varies (Cys/Thr/Ser). A structural alignment of BBSH with Ntn hydrolases using the conserved β-strands as a template reveals that all residues identified as important for catalysis overlap. The BBSH enzyme possesses a catalytic cysteine residue (Cys^4), which of course corresponds to the catalytic nucleophile identified in other enzymes, i.e. PVA Cys^1; penicillin G acylase (PA), Ser^61; cephalosporin acylase (CA), Ser^170; glutamine amidotransferases, Cys^2; glycylasparaginase (AGA), Thr^234; and proteosome, Thr^82.

The α-amino group of this residue is the only candidate close to the nucleophilic sulfur atom that can act as a base. The backbone NH groups of Asn^81 is in an ideal conformation to play the role of the first element of the oxyanion hole (correspondingly PVA Tyr^82; PA Ala^86; CA Val^239; glutamine amidotransferases Gly^103; AGA Gly^235; and proteosome Gly^247). Asn^172 is most likely playing the role of the second contributor to the oxyanion hole (correspondingly PVA Asn^175; PA Asn^B241; CA Asn^413; glutamine amidotransferases Asn^102, AGA Thr^234; and proteosome Ser^824).

Processing Event in Bile Salt Hydrolase—All Ntn hydrolases are synthesized as inactive precursors. In the case of CA and AGA, a single chain precursor is converted into an active heterodimeric form after the precursor undergoes autocatalytic processing. In PVA, the precursor contains a tripeptide at the N terminus, blocking the Cys residue that acts as nucleophile. The autoprocessing is so fast that in the case of wild-type enzyme no processing intermediate can be trapped. However, it is reported that in PVA the Cys to Ala/Gly/Ser mutants lack autoprocessing capability with the result that the first methionine residue is retained in the mutant protein (26). This observation raised the question whether the same phenomenon is occurring in the case of BBSH also. From the crystal structures of both mutants C1A and T2A of BBSH it is inferred that processing is not hampered by these mutations. Thus, in the case of BSH the active form is produced by the removal of the N-terminal formylmethionyl residue. As no further post-translational processing is involved, BSH may prove to be a good model system for further analysis of Ntn hydrolases, as mutants can be assessed without the confusing effects of processing.

Function-Sequence Comparison and Phylogenetic Analysis—

The sequences of an exhaustive list of related proteins available in data bases were aligned (Fig. S1). The evolutionary relationship between these proteins was represented using a dendrogram (Fig. S2). The phylogenetic analysis suggests a diversion of PVA and BSH during evolution despite their apparently conserved structures. From Table 3 we can clearly observe a correlation between sequence homology and enzyme activity. BsPVA shows good activity toward both penV and taurocholic acid substrates. As the sequence departs from PVA toward the BBSH there is a diminishing penV activity and correspondingly increasing BSH activity, whereas the CpBSH sequence represents an intermediate between these two extremes.

Active Site Geometry—The residues reported in the binding pocket of CpBSH are Cys^1, Arg^17, Met^19, Phe^25, Phe^60, Ala^62, Ile^132, Ile^136, and Lys^81. Among these the residues Cys^1, Arg^17, and Ile^132 are strictly conserved in BBSH. The other residues are replaced by Leu^19, Tyr^25, Met^69, Phe^67, Gln^136, and Phe^42, respectively. In CpBSH there are movements of two side chains, those of Trp^143 and Phe^60, upon substrate binding. Residue Trp^143 is conserved (Trp^142 in BBSH), whereas Met^60 replaces Phe^60. A complex of BBSH with the substrate bile salt would have revealed the interaction between the enzyme and the substrate. Unfortunately the mutants did not yield good diffraction quality crystals even after repeated attempts. Attempts to obtain complexes in crystals of BBSH with potential inhibitors or reaction products, by soaking crystals in solutions containing substrates or co-crystallization with inhibitors or substrate, were also unsuccessful.

The nature of the active site was revealed by substrate specificity analysis. The inhibitors showed competitive and mixed type inhibition. The protection provided by these ligands to chemical modification of catalytic residues to various degrees showed that these inhibitors could be binding at the active site, but in an orientation not conductive for activity. Among various inhibitors tested, CA, DCA, and POAA showed good protection toward chemical modification.

The inhibition depended to various extents on the side chain of the inhibitor. The K_i is very much lower for cholic than deoxycholic acid, which is an indication of the strong affinity of B/BBSH toward the oxy substrates. Substitution in the cholic acid ring does not alter the K_i much, which is in good agreement with the previous studies (42, 43) that showed that there was no change in catalytic efficiency on substitution of glycine by other molecules such as taurine, alanine, and 4-amino-3-nitrobenzoic acid etc. The other group of inhibitors in place of cholic acid consists of PAA, POAA, 4-amino-PA, and phenol. The K_i is lower in the case of POAA. There is some evidence to show that variation in the cholic acid side chain affects the catalytic efficiency. It has been noted that substrates lacking a carboxyl group in the side chain are not hydrolyzed. These results indicate that the active site of BSH recognizes only the cholic acid side chain and the α-carboxyl group, whereas allowing various amino acid side chains to go into the binding pocket.

During hydrolysis by BSH the leaving group is glycine or taurine, whereas in hydrolysis by PVA the leaving group is 6-amino-penicillanic acid. Both moieties show little effect when substituted with a wide variety of compounds, indicating that there is no stringent binding condition for these groups. In BSH the cholate and in PVA the PAA groups must be accommodated in the tetrahedral intermediate site within the catalytic site of the enzyme, requiring these groups to possess...
shapes specific for and complementary to the active site binding pockets.

The fluorimetric titration with different substrate and inhibitors provided insight into their binding. The interaction of the enzyme with inhibitor or substrate led to a perturbation in the microenvironment of the tryptophan residue as demonstrated by the quenching or enhancement of tryptophan fluorescence of the enzyme upon binding. The fluorescence enhancement with the addition of substrates like GCA, GDCA, and the quenching of fluorescence with the addition of penV, penG, and POAA was observed. It may be due to the difference in interaction of the inhibitor (PAA group) compared with substrates like GCA, GDCA, and the quenching of fluorescence with the addition of penV, penG, and POAA. Interestingly this tryptophan residue is conserved in true BSHs but not conserved in known PVAs as well as enzymes that have both PVA and BSH activities. We believe that, in addition to other unknown factors, Trp21 also plays a role in selectivity between bile salts and penV as substrates for these hydrolases.

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