J. Biochem. **144,** 295–303 (2008)
doi:10.1093/jb/mvn067

Characterization of Pyridoxal 5'-Phosphate-Binding Domain and Folding Intermediate of *Bacillus subtilis* Serine Hydroxymethyltransferase: an Autonomous Folding Domain

Anant Narayan Bhatt[1,*] and Vinod Bhakuni[2]

[1]Division of Biocybernetics, Institute of Nuclear Medicine and Allied Sciences, Delhi 110 054; and [2]Division of Molecular and Structural Biology, Central Drug Research Institute, Lucknow 226 001, India

Received March 8, 2008; accepted April 17, 2008; published online May 15, 2008

The pyridoxal-5′-phosphate-binding domain (PLPbd) of bsSHMT (*Bacillus subtilis* serine hydroxymethyltransferase) was cloned and over-expressed in *Escherichia coli*. The recombinant protein was solublized, refolded and purified from inclusion bodies by rapid mixing followed by ion exchange chromatography. Structural and functional studies suggested the native form of the domain, which obtained as a monomer and had similar secondary and tertiary structural properties as when present in the bsSHMT. The domain also binds to the PLP however with slightly lesser affinity than the native enzyme. GdmCl (guanidium chloride)-induced equilibrium unfolding of the recombinant PLP-binding domain showed a single monophasic transition which corresponds with the second phase transition of the GdmCl-induced unfolding of bsSHMT. The results indicate that PLPbd of bsSHMT is an independent domain, which attains its tertiary structure before the dimerization of partially folded monomer and behaves as a single cooperative unfolding unit under equilibrium conditions.

Key words: autonomous folding domain, conserved domain, PLP-binding domain, refolding and serine hydroxymethyltransferase.

Abbreviations: bsSHMT, *Bacillus subtilis* SHMT; CD, circular dichroism; eSHMT, E. coli SHMT; GdmCl, guanidium chloride; PLP, pyridoxal-5′-phosphate; SEC, size-exclusion chromatography; SHMT, serine hydroxymethyltransferase and for amino acids single-letter abbreviations have used.

Given the kinetic competition between folding and proteolytic degradation of the nascent polypeptide chain, evolution has found two ways to escape this paradox. First by funnelling the polypeptide through folding pathways (*1–3*) and second, by independent folding by parts of separate domains, supporting multiple nucleation sites and sequential (co-translational) structure formation (*4*, *5*). The latter mechanism is based on the concept that large polypeptide chains consist of domains as stable folding units. Domains represent substructures with specific functional properties with well-defined binding properties for ligands such as substrates, co-enzymes, etc. with the active site of enzyme often being positioned between domains (*6*). However, there is no unequivocal theoretical approach for identifying the boundaries of domains.

Recombinant DNA technology is an alternative and more versatile approach for probing the boundaries of putative structural domains. By using the known structure of the protein as a guide, the separation of protein fragments can be performed at the level of the gene, independent of whether the putative connectors are at the surface of the protein or not. This approach can overcome the truncation of the proteins, by expressing the isolated domains in larger and homogenous amounts in transformed microorganisms (*7*).

Serine hydroxymethyltransferase (SHMT) is a catabolic enzyme that catalyses the removal of one-carbon fragments for the production of nucleotides, methionine, thymidylate, choline and other organic molecules. SHMT also provides glycine for the cell, which through additional processing becomes pyruvate, the core energy molecule for organisms. This process allows microbial cells to convert amino acids, a common carbon substrate in their environment, to simple sugars as a secondary energy source (*8*). Pyridoxal-5′-phosphate (PLP) is a versatile co-factor able to catalyse a spectrum of reactions on a variety of amino acid substrates. Goldsmith and colleagues (*9*) classified PLP-dependent enzymes into five different fold types on the basis of amino acid sequence comparison, predicted secondary structural elements and available three-dimensional structure. SHMT belongs to the type I fold or aspartate aminotransferase family of proteins that is the largest, most diverse, best structurally characterized and found in two different evolutionary lineages, providing examples of convergent enzyme evolution (*10*). These proteins, though genetically dissimilar, have matching secondary and tertiary structures between the subunits of different species (*11*). The final protein complex of the prokaryotes and eukaryotes also differs, with the prokaryotes tending to form tight dimers, whereas the eukaryotes form tetramers (*12*).

*To whom correspondence should be addressed. Tel: 00 91 011 2390 5130, Fax: 00 91 011 2391 9509,
E-mail: anant.bhatt@indiatimes.com

Each subunit of bsSHMT folds into two domains; a large PLP-binding domain and a small C-terminal domain. The N- and C-terminal parts of the chain do not have a common fold within this class. However, the central and largest part that forms α/β barrel domain binds the co-factor PLP. All the PLP-dependent enzymes are predicted to possess an α/β barrel motif for binding the co-factor. Hence, this domain is the most conserved part and has the same fold in entire family, even though some members of the family are least similar in amino acid sequence (*13*–*16*). The large PLP-binding domain comprises of 214 amino acid residues starting from 63 to 276 (numberings as for *Bacillus stearothermophilus* serine hydroxymethyltransferase, bstSHMT; PDB id, 1KKJ) and includes all side chains that are important for the binding of PLP (*13*).

There are a few reports about the independent PLP-binding domain of *Escherichia coli* aspartate aminotransferase and role of PLP in the folding of SHMT (*17*, *18*) but, not much have been reported about the role of PLP-binding domain in the folding and stability of SHMT. Moreover, the native bsSHMT appears to follow a three-state unfolding transition involving a compact intermediate (*14*). The availability of pure, homogenous independent domain would allow the characterization of intermediate state and will help in assessment of the independent folding capability of co-factor-binding domain of bsSHMT.

In the present study, the recombinant PLP-binding domain of bsSHMT was produced based solely on the structural boundaries of the domain. We report on the cloning, over-expression, refolding, purification, biochemical as well as structural characterization of the recombinant PLP-binding domain. Structural changes associated with temperature and GdmCl-induced unfolding of the domain was studied and compared to those of native bsSHMT.

EXPERIMENTAL PROCEDURES

Construction of PLP-Binding Domain Expressing Plasmid—The 650 bp gene encoding 214 aa (G63–D276)-long PLP-binding domain was PCR (polymerase chain reaction) amplified from the full-length gene of bsSHMT (pET21d-bsSHMT) using the following primers: sense primer, 5′-CGCGGATCCGGATGTGAGCACGTCG ACGTC-3′ and anti-sense primer, 5′-GGGGTACCCTAGT CCTGCAATACTTCACCGAATGA-3′, containing BamH1 and Kpn1 restriction sites (underlined nucleotides) at 5 and 3′, respectively. The PCR reaction was carried out for 30 cycles in a total volume of 50 μl with 1 U of *Taq* polymerase and thermocycler condition used was 95°C, 1 min denaturation; 57°C, 1 min annealing; 72°C, 2 min amplification, respectively. The fragment was cloned into vector (pQE30) between BamHI and KpnI sites and the DNA sequence was confirmed by sequencing. The obtained plasmid (pQE30plpbd) was introduced into *E. coli* BL21 (DE3) cells for protein expression and was induced at OD_{600} of 0.6 by adding a final concentration of 0.5 mM IPTG (isopropyl β-D thiogalactopyranoside). The cells were further grown for 3 h at 37°C before harvesting. The cell pellet was re-suspended in 50 mM Tris buffer pH 7.4 containing 1 mM EDTA and 2 mM β-mercaptoethanol and stored at –80°C for further use.

Isolation, Solubilization and Refolding of Recombinant PLPbd Inclusion Bodies—The frozen cell pellets were freeze-thawed (thrice) and after a mild sonication cell lysate was centrifuged at 24,000*g* for 15 min. Pellet was washed with distilled water twice to remove salts. The final sediment containing inclusion bodies was used for solubilization. Inclusion bodies were solubilized (1 mg/ml) in 50 mM potassium phosphate buffer pH 7.4 containing 6 M urea, and 0.5 M NaCl for 1 h at 4°C and centrifuged for 30 min at 24,000*g*. Five millilitres of supernatant containing the solubilized domain was added drop by drop to 100 ml of stirring potassium phosphate buffer (50 mM, pH 7.4 containing 1 mM EDTA, 2 mM β-mercaptoethanol, 0.1 mM PLP and 0.1 M NaCl). This refolded protein solution was centrifuged at 24,000*g* for 30 min and the supernatant was dialysed against the potassium phosphate buffer (50 mM, pH 7.4 containing 1 mM EDTA and 2 mM β-mercaptoethanol) with three changes of buffer.

Purification of Recombinant PLP-Binding Domain—After dialysis the refolded mixture, which had only 10% protein contaminants was loaded on MonoQ column (GE Healthcare, Sweden) equilibrated with the same buffer (50 mM, pH 7.4 containing 1 mM EDTA and 2 mM β-mercaptoethanol). The protein was eluted as a linear gradient with final buffer containing 1 M NaCl. The purity of the obtained protein was checked using SDS–PAGE and was found to be >98% pure. The yield by mass of refolded protein from a pellet was found 20–25%.

CD (Circular Dichroism) Measurements—CD measurements were made with a Jasco J810 spectropolarimeter calibrated with ammonium (+)-10-camphorsulphonate. The results are expressed as relative ellipticity and plotted as percentage values. The CD spectra were measured at an enzyme concentration of 3 and 15 μM for far- and near-UV or visible CD measurements, respectively. Far- and near-UV measurements were made in 2 and 10-mm path length quartz cell, respectively at 25°C. The values obtained were normalized by subtracting the baseline recorded for the buffer having same concentration of denaturant under similar conditions. Thermal denaturation experiments were performed by increasing the temperature from 20 to 95°C. The observed ellipticities at 222 nm were recorded against the increasing temperature.

Synthesis of PyP-SHMT—The reduction of PLP aldimine was achieved according to the procedure of Cai and Schirch (*19*). In brief, cells were dialysed with 0.1% $NaCNBH_3$ in 50 mM phosphate buffer, pH 7.4 containing 1 mM EDTA and 2 mM β-mercaptoethanol for 12 h. Further, excess $NaCNBH_3$ was removed by dialysing the protein against same buffer for 24 h.

Limited Proteolysis—The native and 1 M GdmCl-treated samples were subjected to limited proteolysis with α-chymotrypsin. The protein (1 mg/ml) was incubated in presence and absence of GdmCl for 4 h at 4°C before adding the α-chymotrypsin (protease: protein ratio of 1:100). Proteolysis was carried out for 5 min at same temperature in a 100 μl reaction volume. Reaction was

stopped with 2 mM PMSF and the mixture was analysed on 12% SDS–PAGE.

Size-Exclusion Chromatography—Gel filtration experiments were carried out on Superdex 200HR 10/30 column (exclusion limit 600 kDa for proteins) on AKTA FPLC (GE Healthcare, Sweden), pre-equilibrated and run with phosphate buffer (50 mM, pH 7.2 containing 1 mM EDTA, 2 mM β-mercaptoethanol and 150 mM NaCl). The protein sample 200 μl was loaded on the column and run at 25°C with the flow rate of 0.3 ml/min and continuous detection at 280 nm. The apparent molecular masses and Stoke's radii of the proteins were determined from the calibration curves for proteins of known molecular mass and Stoke's radius (BSA, 66.3 kDa; 33.9 A°; ovalbumin, 43.5 kDa; 31.2 A°; myoglobin, 16.9 kDa; 20.2 A° and cytochrome c, 11.7 kDa; 17.0 A°).

RESULTS AND DISCUSSION

The PLP-binding domain in isolation retains the fold pattern and symmetry compared to the native bsSHMT (Fig. 1; PDB id, 1KKJ). We report first time the characterization of an isolated co-factor-binding domain of any SHMT.

Refolding of Inclusion Bodies—The over-expression of recombinant proteins in the reducing environment of *E. coli* cytosol frequently leads to intra-cellular accumulation of its aggregated form (*20*). Mostly the solublization of these insoluble aggregates followed by the removal of effective concentration of denaturant is necessary for refolding and in obtaining the soluble and active form of the protein. We refolded the inclusion bodies into soluble native form and purified (98%) efficiently (Fig. 2).

The refolding of the inclusion bodies was observed by monitoring the change in far-UV CD-spectra for secondary structure. Figure 3A shows the spectra of inclusion bodies solublized in 6.0 M urea as well as those of 20 times diluted and refolded protein sample. The CD spectra of diluted fraction of proteins suggest that the refolded domain has significant amount of secondary structure. Refolding of the solubilized inclusion bodies of the isolated domain was also examined on size-exclusion column by monitoring the hydrodynamic radii of the refolded fragment. The inclusion bodies were solubilized in 6.0 M urea containing buffer and then the fraction was diluted twenty times, centrifuged at 24,000*g* for 10 min. Supernatant, with protein concentration 0.2 mg/ml was loaded on the Superdex S-200 column, equilibrated with phosphate buffer without urea, in the presence and absence of co-factor PLP, it was eluted as a single peak without any visible aggregates in the chromatogram (Fig. 3B, and inset). The elution volume, compared with standard protein elution profile, suggests that the refolded protein has 25 kDa molecular weight which is similar to the theoretical molecular weight of the domain; hence it suggests that the fragment folded successfully on dilution in the column. Under similar conditions, in the presence of PLP which was monitored at 425 nm together with $A_{280\,nm}$ the protein was eluted again at the same retention volume with another peak, which denote the absorbance of PLP together with the protein (Fig. 3B and inset). Elution of refolded domain at the same retention volume in the presence and absence of PLP shows that the co-factor does not play any role in the folding of PLP-binding domain, which is in line with earlier observation on eSHMT (*E. coli* SHMT) (*21*). Binding of the co-factor PLP to the domain also suggests that the refolded domain attains its native fold during refolding from inclusion bodies. The results presented above indicate clearly that the fragment over-expressed represents the independent folding domain and its efficient refolding may be due to the high stability of this PLP-binding domain. The over-expressed protein have formed inclusion bodies inside the bacterial cell probably, because of high concentration of over-expressed protein and some exposed hydrophobic patches that would have masked by the other domain in the native molecule.

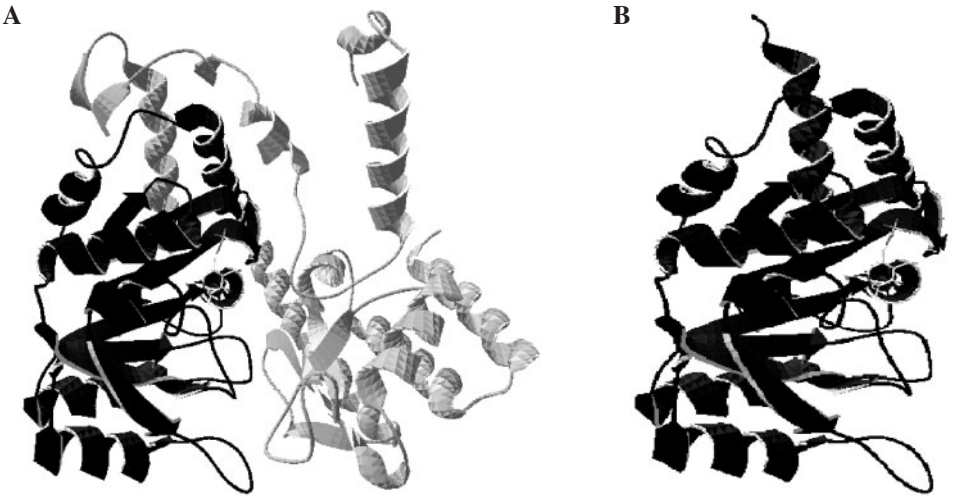

Fig. 1. **The picture is showing the bsSHMT.** (A) and the isolated PLP-binding domain (B). In the fig. A, N-terminal and C-terminal portion is shown in light grey colour and co-factor-binding domain is in black colour. Figure B is the isolated part of bsSHMT from residue G63 to D276. The pictures were produced in program 'SPDBV' by using bstSHMT crystal structure as template (PDB id, 1KKJ) (*13*).

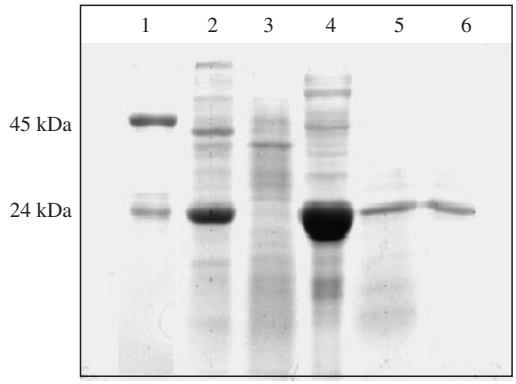

Fig. 2. The gel photograph is showing molecular weight markers (lane 1). Lanes 2–4 is showing pellet of cell lysate having protein as inclusion bodies, supernatant of cell lysate and inclusion bodies solubilized in 6 M urea, respectively. Lanes 5 and 6 are representing refolded and purified soluble domain, respectively.

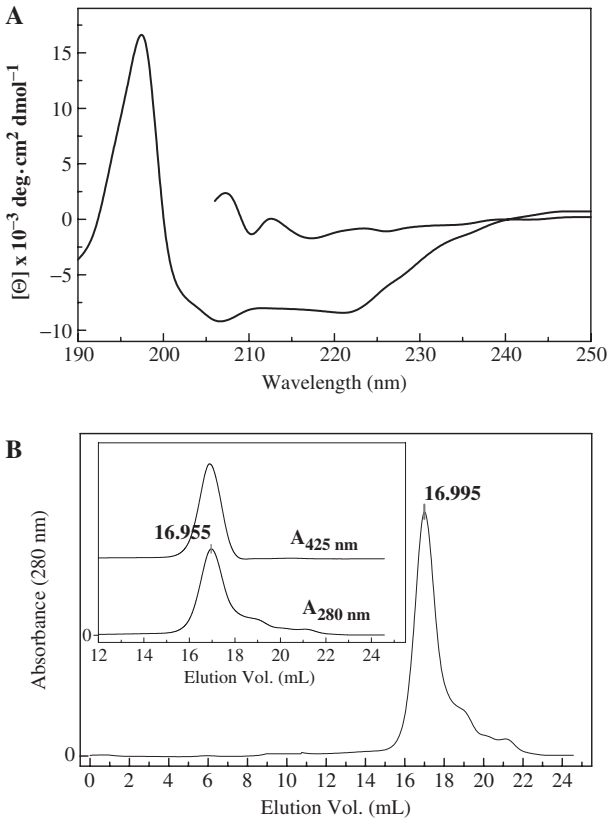

Fig. 3. **Refolding of inclusion bodies.** (A) The Far-UV CD spectra represents the inclusion bodies, solubilized in 6 M urea (curve 1) and 20 times diluted and refolded PLP-binding domain (curve 2). The chromatograms are showing refolding of inclusion bodies in to folded domain, in the absence (B) and in the presence **(inset B)** of PLP (co-factor), monitored by size-exclusion chromatography.

The successful refolding of independent domain indicates that this method of refolding is useful for obtaining the recombinant isolated domains in soluble form and can be proven a great approach in deciphering the functional and structural roles of individual domains in multi-domain proteins.

Structural Characterization of Isolated Domain—One of the serious problems in interpreting the data obtained with protein fragments is that the 'nativeness' of the fragments is always doubtful. Even if the fragments are shown to be fully compact and globular there is no guarantee that they will behave in exactly the same way, as they would have done when they were part of the intact protein molecule. This made us to look for the structural characterization, which will be helpful in drawing any conclusion about the nativeness of the refolded recombinant domain.

The effect of the removal of N- and C-terminal extensions from the molecule was observed on the secondary and tertiary structure of the PLP-binding domain.

Secondary Structural Characterization by Far-UV CD Spectroscopy—The far ultraviolet CD spectra for the recombinant domain were examined to obtain the information on the conformation of the recombinant domain's polypeptide backbone. Figure 4A summarizes the comparison between the secondary structure of the isolated domain and wild-type bsSHMT. For the bsSHMT, far-UV CD spectrum with a positive ellipticity maximum at 193 nm and negative ellipticity maxima at 208 and 222 nm, typical of proteins with high α-helical content was observed. The homology model of the domain suggests that the isolated domain folds into a αβα structure consisting of eight-stranded β-sheet flanked by α-helices on both sides (Fig. 1B). Secondary structural analysis from the bsSHMT model suggests that the domain has almost 40% of its secondary structure as α-helix. The observed spectra showed maxima at 196 nm, minima at 209 nm and broad minima centred at 220 nm, which is similar to those for proteins with mixed type of secondary structures. This implies that the recombinant domain has similar secondary structural component as predicted by the homology model of the native protein bsSHMT and have folded conformation.

Tertiary Structural Characterization by Near-UV CD Spectroscopy—The tertiary structural information of isolated domain was also obtained using CD spectroscopy. Figure 4B represents comparison of the near-UV and visible CD spectra of the bsSHMT and recombinant PLP-binding domain. For both bsSHMT and isolated domain, strong near-UV and visible CD signal were observed suggesting the presence of buried tyrosine residues and bound PLP in the proteins (*22*, *23*). The existence of peak in near-UV region indicates that some or most of the tyrosine residues of recombinant PLP-binding domain are buried in specific and tightly packed environments and it was proposed that maxima at 275 nm can serve as markers for the native fold (*24*). Furthermore, compared to the wild-type proteins, the polarization spectra of holo domain gave little blue shift in visible CD signal. This shift in the spectra is probably because of the relatively free orientation of the PLP molecule in the domain whereas, in the wild-type bsSHMT, PLP is bound to both domain and dimer interface and cannot rotate freely. The ellipticity change of recombinant holodomain in near-UV CD, due to buried tyrosine residues suggests an asymmetric environment of

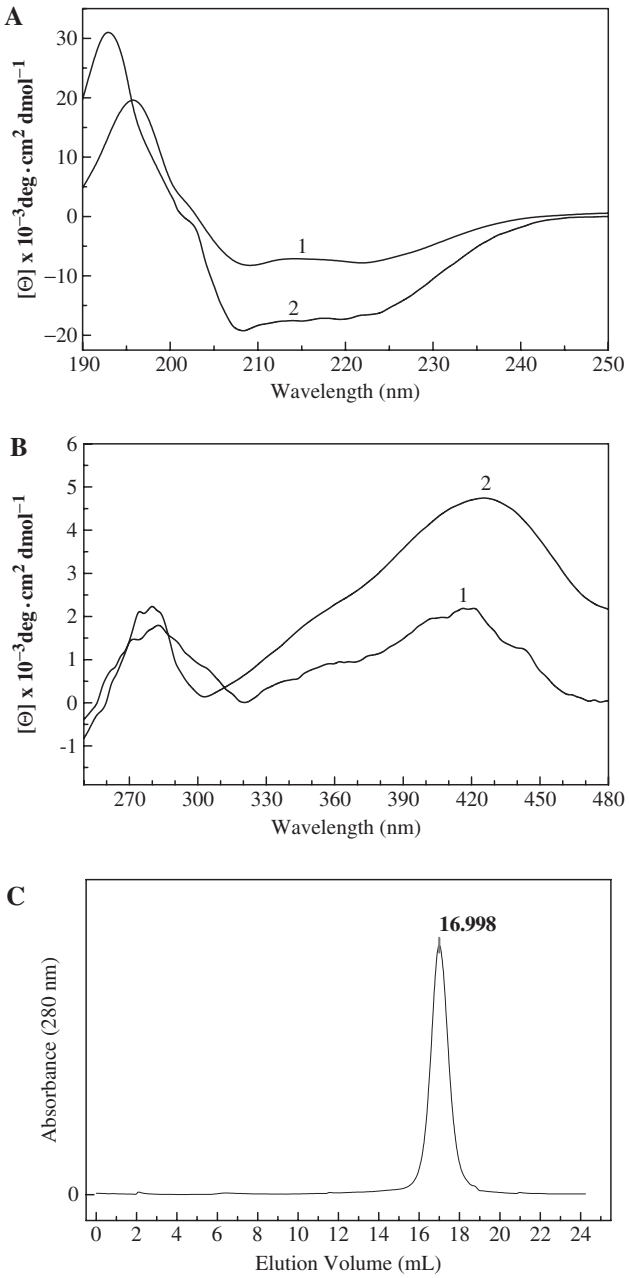

Fig. 4. **Structural characterization of recombinant PLPbd.** (A) Far-UV CD spectra of, the recombinant isolated domain (spectrum 1) and dimeric bsSHMT (spectrum 2). (B) Near- and Visible-CD spectra of recombinant PLP-binding domain (spectrum 1) and dimeric bsSHMT (spectrum 2). (C) The chromatogram is showing the elution profile of refolded and purified recombinant PLP-binding domain of bsSHMT. The number on peak is showing the retention volume.

the chromophore that is similar to the native bsSHMT. The differences observed in the secondary structure content could be due to variable amounts of structures present between the native enzyme and the isolated domain.

Characterization of Oligomeric State—The oligomeric state of recombinant domain in solution was investigated on size-exclusion chromatography. Gel filtration chromatogram (Fig. 4C) of the refolded isolated fragment represents the elution profile. The apparent molecular mass of the peak was estimated to be ~25 kDa. This information suggests that the domain is monomeric even at the concentration of 1 mg/ml (40 μM) and at this concentration protein was not showing any aggregation on storage up to 1 month.

Functional Characterization—SHMT (EC 2.1.2.1) contains covalently bound pyridoxal-P, which forms an internal aldimine with the ∈-amino group of lysine. This enzyme is a member of the α-class of pyridoxal-5′-phosphate enzymes, catalyses the reversible interconversion of serine and tetrahydrofolate (THF) to glycine and 5, 10 methylene tetrahydrofolate, changing the chemical bonding at the C^{α}–C^{β} bond of the serine side chain, mediated by the pyridoxal phosphate cofactor with the help of another coenzyme H_4-folate (*25*).

The specific binding of PLP can be detected by UV-visible absorption spectroscopy. The protein bound PLP gives absorption maxima at 340 and 425 nm, which are generally ascribed to enolimine and ketoenamine tautomers, respectively, of the Schiff base formed between PLP and a lysine residue of the protein. But in most PLP-binding proteins, the ketoenamine form that absorbs at around 425 nm dominates (*17*, *26*). The co-enzyme PLP itself can bind covalently and form external aldimine complex with glycine and a marked and rapid increase in the intensity of the yellow colour of the solution together with the increase in absorption can be observed. This increase and red shift in absorbance is because of the Schiff's base formation between free PLP and glycine (*25*).

Since, the residues which constitute the active site in the native enzyme not only come from different parts of the polypeptide chain but also from other polypeptide chain (B chain) of dimer, it was expected that the isolated co-factor-binding domain would not be active, although it binds PLP. All the residues those are responsible for the binding of co-factor PLP lie between G63 and D276 of the domain, except Y51 and G257 (B chain), which come from the other chain of the dimer in the native molecule. The numberings are according to the crystal structure of bstSHMT (*13*) which have strong homology and identity 96 and 86%, respectively in this region. The presence of most of the PLP-binding residues in the domain ensures that the co-factor PLP can bind to the molecule. And the binding of the co-factor to the recombinant PLP-binding domain was further verified by the strong absorption of domain bound PLP at 425 nm in absorption spectra of the holodomain (curve 2 in Fig. 5A), the spectrum of the holodomain solution was subtracted by that of the free PLP. The apo-form of the domain, which was obtained by incubating the holodomain with 10 mM hydroxylamine in phosphate buffer was further incubated with 2 μM PLP per 1 μM of the domain and then was dialysed against the buffer to remove unbound and excess PLP. The proper binding of PLP to the recombinant domain guaranteed by the absorption of holodomain at 425 nm, like the native molecule; suggests that the domain is in native and functional conformation. As discussed above, holodomain can also bind the amino acid glycine through PLP, which is also the substrate for

the enzyme SHMT and most of the residues which bind the substrate glycine are present in this isolated PLPbd (*13*). Hence, the holodomain was incubated with 50 mM glycine and spectra were recorded between 550 and 380 nm (Fig. 5A). The curve 3 represents the enhanced intensity of absorption of the complex of holodomain with glycine. Since, the free PLP present in the solution can also form the complex with the excess of glycine in the reaction buffer; the spectrum was subtracted with equimolar free PLP and glycine complex and presented as curve 4. The red shift (Fig. 5A, curve 4) in the absorption of the bound PLP indicates that the domain bound PLP molecule formed complex with the substrate glycine (*25*).

The holodomain was reduced with sodium cyanoborohydride ($NaCNBH_3$) to determine weather PLP is bound covalently as a Schiff's base. As expected, the absorbance maximum at 425 nm was shown blue shift in reduced protein as a single and relatively broader peak at around 355 nm (Fig. 5B), which is characteristic of ∈-(phosphopyridoxyl) lysine (*17*). This result showed that borohydride reduced the aldimine linkage to yield the aldamine bond that usually absorbs in the range of 325–340 nm in PLP-binding enzymes but broader absorbance of pyridoxamine-5′-phosphate (reduced aldimine) with peak centered around 355 nm is close to the typical values for the deprotonated Schiff base (360–380 nm); whereas, the deprotonated Schiff base should not be formed under the described condition. Deprotonation of Schiff base usually occurs because of the movement of the aldimine from hydrophobic environment to less hydrophobic environment (*27*). And this could be possible because of the relatively free orientation of the PLP molecule in the domain.

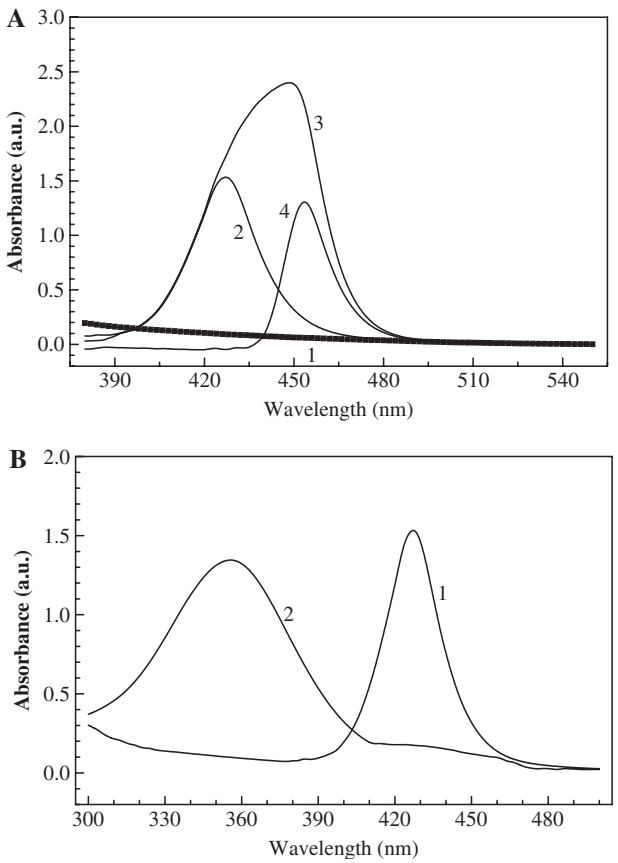

Fig. 5. **Functional characterization of recombinant PLPbd.** (A) The curve 1 represents the absorption spectra of apodomain. Curve 2 shows the absorption spectrum of holodomain, which is subtracted with the spectrum of 5 μM PLP to negate the presence of free PLP. The curve 3 represents the absorption spectra of glycine complex with holodomain together with free PLP-glycine complex and curve 4 is representing the pure glycine-holodomain complex obtained by subtracting the equimolar free glycine PLP complex without PLPbd. (B) The diagram is showing the spectroscopic changes before (curve 1) and after (curve 2) reduction of the PLP in holo-domain with $NaCNBH_3$. All the spectral measurements for functional characterization of PLPbd, were carried out at 5 μM protein concentration.

The absorption spectra of holodomain and its complex formation with glycine shows that the molecule is in its native fold and these results together with secondary and tertiary structural characterization (Figs 4 and 5) of the isolated and refolded domain indicate that the PLP-binding domain is autonomous and independent folding domain.

Temperature Denaturation of the Isolated Domain— The thermo-stability of recombinant PLP-binding domain was examined based on the residues molar ellipticity of the polypeptide chain at increasing temperatures. In the far-UV region, the CD spectrum of native isolated domain and bsSHMT both shows the presence of substantial amount of ellipticity at 222 nm. Hence, loss of CD signal at 222 nm was taken as a measure of the unfolding of the independent domain with temperature (Fig. 6, curve 1). A broad sigmoidal transition between 30°C and 65°C having an apparent T_m of about 48°C was observed for the native dimeric bsSHMT molecule (Fig. 6, curve 2). This indicates that bsSHMT is a non-co-operative molecule which starts losing the structure from very low temperature (30°C) and loses most of its secondary structure at relatively high temperature. Whereas, PLPbd is stable at temperature up to 45°C,

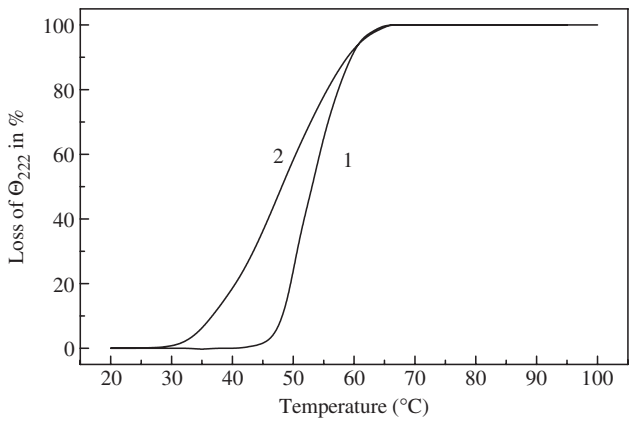

Fig. 6. **Thermal denaturation of recombinant domain and bsSHMT measured by loss of CD ellipticity at 222 nm.** A linear extrapolation of baselines in pre and post-transitional regions was used to determine the fraction-unfolded protein within the transition region by assuming a two-state mechanism of unfolding. The curves 1 and 2 represent data for PLP-binding domain and bsSHMT, respectively.

the temperature at which native bsSHMT already lost >40% structure. This is an interesting observation that coincides with the data in which the intermediate populated in the GdmCl-induced folding pathway of bsSHMT was 40% less structured and was characterized with 60% residual secondary structure (*14*). On the other hand, isolated domain shows relatively cooperative unfolding against increasing temperature with a sigmoidal transition between 45°C and 65°C having an apparent T_m of about 53°C, demonstrating that the major part of the protein constituted by this domain is relatively resistant to thermal unfolding. The T_m of the isolated domain (53°C) was found close to that the T_m (54°C) of the 1 M GdmCl stabilized intermediate, characterized in folding pathway of bsSHMT in Differential Scanning Calorimetry (*14*).

The temperature induced unfolding of recombinant domain in comparison with the thermal unfolding of bsSHMT (*14*), suggest that the PLP-binding domain is the most stable and co-operative unit of the native molecule.

GdmCl-Induced Unfolding of PLP-Binding Domain—The conformational stability of proteins can be measured by equilibrium unfolding studies using protein denaturants like GdmCl. Analysis of the solvent denaturant curves using denaturants can provide a measure of the conformational stability of the proteins (*28*). Structural characterizations of the partially folded intermediates stabilized during denaturant-induced folding/unfolding of the proteins have provided significant inputs on the forces that stabilize these folded intermediates of protein.

Figure 7A shows the change in mean residual ellipticity at 222 nm with increasing concentration of GdmCl for the bsSHMT and its isolated recombinant PLP-binding domain. The GdmCl-induced unfolding of the recombinant domain showed two-state transition with a slight decrease in the structure for upto 1 M GdmCl. The transition was observed between 1 and 2 M GdmCl, and the domain is almost unfolded with ~80% loss of the secondary structure at 2 M GdmCl. The transition mid point (Cm value) for domain was found to be about 1.4 M which is quite similar to the second transition (1.44 M) of the biphasic bsSHMT denaturation. The far-UV CD spectra of the intermediate stabilized at 0.65–1 M GdmCl possessed about 64% of the native CD spectral amplitude. On the other hand, the far-UV CD spectra of 1 M GdmCl stabilized intermediate of the bsSHMT and native isolated domain both shows common and broad minima at around 220 nm (Fig. 7B, curve 2). Although, both these spectra presented in Fig. 7B were taken in two different conditions, their similarity shows that the partially structured intermediate formed in the equilibrium-unfolding pathway of bsSHMT has similarity with fully structured and isolated recombinant PLP-binding domain. The results obtained from the GdmCl-induced unfolding of dimeric bsSHMT and its recombinant PLP-binding domain suggest that the intermediate stabilized in the GdmCl-induced unfolding pathway of dimeric bsSHMT, is the structured co-factor-binding apodomain with unstructured residual part of the monomeric chain of bsSHMT molecule. To further verify this fact and obtain the structured

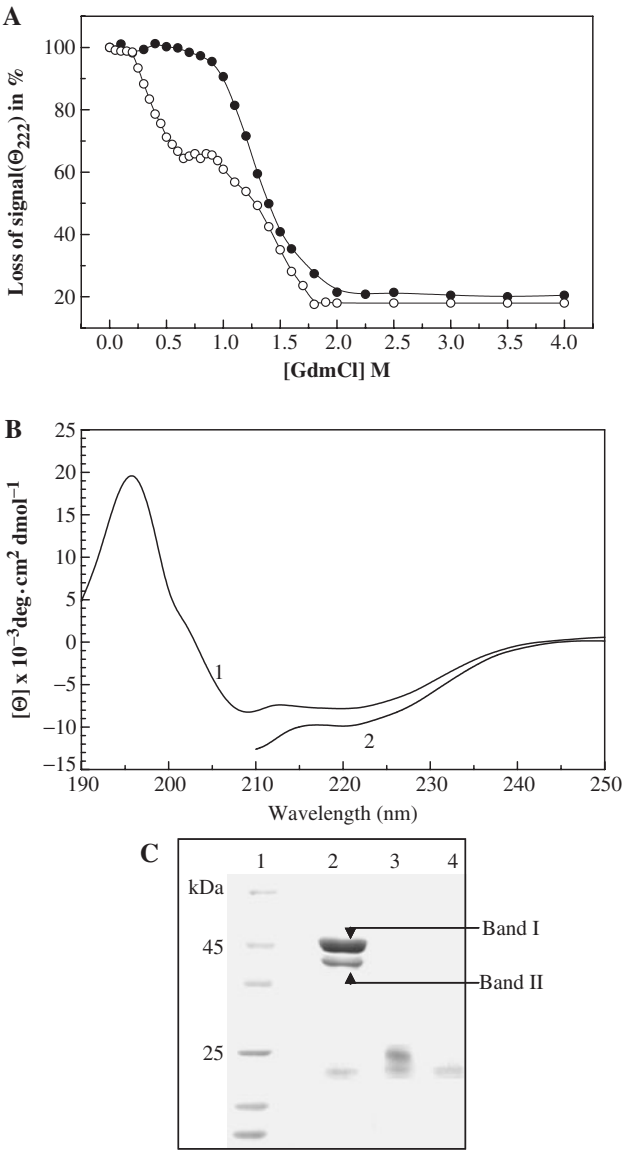

Fig. 7. **GdmCl-induced equilibrium unfolding of bsSHMT and its co-factor-binding domain as studied by CD-spectroscopy.** (A) GdmCl-dependent unfolding of bsSHMT (open circle) and isolated domain (filled circle) monitored at 222 nm. The data are represented as a percentage of ellipticity at 222 nm taking the value for the native enzyme to be 100%. (B) The diagram shows comparison of CD spectra of isolated domain (curve 1) and bsSHMT in its intermediate state at 1 M GdmCl (curve 2). (C) Picture shows, SDS–PAGE analysis of bs-SHMT on limited proteolysis with -chymotrypsin. The protein to protease ratio was 100:1. In the figure the lanes 1–4 represent molecular weight markers, -chymotrypsin treated bsSHMT, -chymotrypsin treated 1.0 M GdmCl stabilized bsSHMT and equal amount of protease, respectively.

portion of the intermediate, limited proteolysis of dimeric bsSHMT incubated with 1.0 M GdmCl, using α-chymotrypsin was carried out.

Figure 7C, shows the SDS–PAGE profile of native and GdmCl incubated bsSHMT on proteolysis with α-chymotrypsin. For bsSHMT two major protein bands, corresponding to the native protein (Band I) and other to

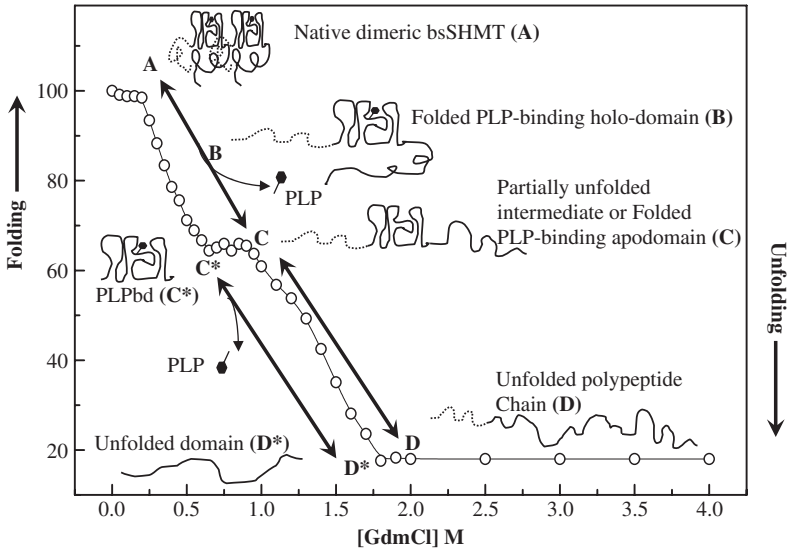

Fig. 8. **Schematic representation of role of PLP-binding domain in folding pathway of dimeric bsSHMT.**

the proteolysed fragment (Band II) of the bsSHMT along with several minor, low molecular weight protein bands were observed on treatment of enzyme with α-chymotrypsin. The N-terminal sequencing of the band II suggests that it is devoid of 47 amino acid from N-terminal (*22*). However, 1 M GdmCl incubated bsSHMT gives a major band of about 25 kDa on limited proteolytic digestion with α-chymotrypsin. Subsequent, N-terminal sequence analysis of this proteolysed product indicates that N-terminus of this band corresponds to amino acid number 83. This result also suggests that the proteolysis of intermediate state populated during the unfolding pathway of bsSHMT at low GdmCl concentration has major part (about 50%) of the native as structured, which resists the proteolytic digestion and corresponds to the major and central domain of the polypeptide chain that is the PLP-binding domain.

The GdmCl-induced unfolding of isolated domain was found to be co-operative, where there was no stabilization of any partially unfolded intermediate. GdmCl probably denatured the domain directly by solvating the core of the molecule and unfolded it completely. This information from the denaturation of isolated recombinant PLP-binding domain can be well correlated with the unfolding of dimeric bsSHMT, which was dissociated probably because of disturbance in ionic interactions on the dimeric interface and populated with the partially unstructured and hydrophobic intermediate (*14*). Since, it has been already shown that the populated intermediate has structured PLP-binding domain (Fig. 7C) along with a residual unstructured part, it can be said that the intermediate was further denatured due to the solvation of hydrophobic core of the domain.

The present study and the previous results (*14*) suggests that the PLP-binding domain is the most stable part of bsSHMT, which attains its tertiary structure very early in the folding pathway and may acts as a core of the dimeric bsSHMT (Fig. 8). This observation has genetic importance because the region which binds PLP is mostly conserved in the entire aspartate aminotransferase family and up to some extent in the PLP-binding proteins (*9*, *10*).

Dr C.M Gupta is thanked for constant support provided during the studies. A.N.B. wants to thank Dr. Md. Sohail Akhtar and Council of Scientific and Industrial Research.

REFERENCES

1. Rose, G.D., Fleming, P.J., Banavar, J.R., and Maritan, A. (2006) A backbone based theory of protein folding. *Proc. Natl Acad. Sci. USA* **103**, 16623–16633
2. Karplus, M. and Shakhnovich, E. (1992) *In* Protein Folding (Creighton, T.E., ed.) Freeman, New York
3. Dill, K.A., Bromberg, S., Yue, K., Fiebig, K.M., Yee, D.P., Thomas, P.D., and Chan, H.S. (1995) Principles of protein folding--a perspective from simple exact models. *Protein Sci.* **4**, 561–602
4. Wetlaufer, D.B. (1973) Nucleation, rapid folding, and globular intrachain regions in proteins. *Proc. Natl Acad. Sci. USA* **70**, 697–701
5. Goldberg, M.E. (1991) Investigating protein conformation, dynamics and folding with monoclonal antibodies. *Trends Biochem. Sci.* **10**, 358–362
6. Rossmann, M.G. and Argos, P. (1981) Protein folding. *Annu. Rev. Biochem.* **50**, 497–532
7. Minard, P, Hall, L., Betton, J.M., Missiakas, D., and Yon, J.M. (1989) Efficient expression and characterization of isolated structural domains of yeast phosphoglycerate kinase generated by site-directed mutagenesis. *Protein Eng.* **1**, 55–60
8. Pascarella, S., Schirch, V., and Bossa, F. (1993) Similarity between serine hydroxymethyltransferase and other pyridoxal phosphate-dependent enzymes. *FEBS Lett.* **331**, 145–149
9. Grishin, N.V., Phillips, M.A., and Goldsmith, E.J. (1995) Modelling of the spatial structure of eukaryotic ornithine decarboxylase. *Protein Sci.* **4**, 1291–1304
10. Christen, P. and Mehta, P.K. (2001) From cofactor to enzymes. The molecular evolution of pyridoxal-5'-phosphate-dependent enzymes. *Chem. Rec.* **1**, 436–447

11. Kazanina, G., Radaev, S., Wright, H.T., and Schirch, V. (1998) Crystal forms and subunit stoichiometry of serine hydroxymethyltransferase. *J. Struct. Biol.* **123**, 169–174
12. Rao, N.A., Ambili, M., Jala, V.R., Subramanya, H.S., and Savithri, H.S. (2003) Structure- function relationship in serine hydroxymethyltransferase. *Biochim. Biophys. Acta* **1647**, 24–29
13. Trivedi, V., Gupta, A., Jala, V.R., Saravana, P., Rao, J., Rao, N.A., Savithri, H.S., and Subramanya, H.S. (2002) Crystal structure of binary and ternary complexes of serine hydroxymethyltransferase from Bacillus sterothermophilus. *J. Biol. Chem.* **277**, 17161–17169
14. Bhatt, A.N., Prakash, K., Subramanya, H.S., and Bhakuni, V. (2002) Different unfolding pathways for mesophilic and thermophilic homologues of Serine hydroxymethyltransferase. *Biochemistry* **41**, 12115–12123
15. Smith, D.L., Almo, S.C., Toney, M.D., and Ringe, D. (1989) 2.8-A-resolution crystal structure of an active-site mutant of aspartate aminotransferase from Escherichia coli. *Biochemistry* **28**, 8161–8167
16. Jager, J., Kohler, E., Tucker, P., Sauder, U., Housley-Markovic, Z., Fotheringham, I., Edwards, M., Hunter, M., Kirschner, K., and Jansonius, J.N. (1989) Crystallization and preliminary X-ray studies of an aspartate aminotransferase mutant from Escherichia coli. *J. Mol. Biol.* **209**, 499–501
17. Herold, M., Leistler, B., Hage, A., Luger, K., and Kirschner, K. (1991) Autonomous folding and coenzyme binding of the excised pyridoxal 5'-phosphate binding domain of aspartate aminotransferase from Escherichia coli. *Biochemistry* **30**, 3612–3620
18. Stark, W., Kallen, J., Markovic, H.-Z., Fol, B., and Jansonious, J.N. (1991) *Proceedings of the International Symposium on Vitamin B6.* Pergamon Press, Oxford, England
19. Cai, K. and Schirch, V. (1996) Structural studies on folding intermediates of Serine hydroxymethyltransferase using fouriar resonance energy transfer. *J. Biol. Chem.* **271**, 27311–27320
20. Misawa, S. and Kumagai, I. (1999) Refolding of therapeutic proteins produced in Escherichia coli as inclusion bodies. *Biopolymers* **51**, 297–307
21. Fu, T., Boja, S.E., Safo, M.K., and Schirch, V. (2003) Role of proline residues in the folding of serine hydroxymethyltransferase,. *J. Biol. Chem.* **278**, 31088–31094
22. Bhatt, A.N., Khan, M.Y., and Bhakuni, V. (2004) The C-terminal domain plays a key role in stabilization of the quaternary structure and cooperative unfolding of dimeric SHMTs: domain swapping studies with enzymes having high sequence identity. *Prot. Sci.* **13**, 2184–2195
23. Strickland, E.H. (1972) Interactions contributing to the tyrosyl circular dichroism bands of ribonuclease S and A. *Biochemistry* **11**, 3465–3474
24. Pain, R. (1996) Determining the CD spectrum of a protein in Current Protocols in Protein Science (Coligan, J.E., Dunn, B., Ploegh, H.L., Speicher, D.W., and Wingfield, F.T., eds.) pp. 723–761, John Wiley & Sons
25. Blakley, R.L. (1955) The interconversion of serine and glycine: participation of pyridoxal phosphate. *Biochem. J.* **61**, 315–323
26. Kallen, R.G. and Korpela, T. et al. (1985) In Transaminases (Christen, P. and Metzler, D.E., eds.) pp. 37–108, Wiley, New York
27. Olmo, M.T., Sánchez-Jiménez, F., Medina, M.A., and Hayashi, H. (2002) Spectroscopic analysis of recombinant rat histidine decarboxylase. *J. Biochem.* **132**, 433–439
28. Pace, C.N. (1986) Determination and analysis of urea and guanidine hydrochloride denaturation curves. *Methods Enzymol.* **131**, 266–280