Role of Proline Residues in the Folding of Serine Hydroxymethyltransferase*

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Previous studies on the folding mechanism of Escherichia coli serine hydroxymethyltransferase (SHMT) showed that the final rate determining folding step was from an intermediate that contained two fully folded domains with N-terminal segments of approximately 55 residues and interdomain segments of approximately 50 residues that were still solvent exposed and subject to proteolysis. The interdomain segment contains 3 Pro residues near its N terminus and 2 Pro residues near its C terminus. The 5 Pro residues were each mutated to both a Gly and Ala residue, and each mutant SHMT was purified and characterized with respect to kinetic properties, stability, secondary structure, and folding mechanism. The results showed that Pro216 and Pro258 near the N terminus of the interdomain segment are not critical for folding, stability, or activity. The P216A mutant also retained most of the characteristics of the native enzyme, but its folding rate was altered. However, the P216G mutant was severely compromised in folding into a catalytically competent enzyme. Mutation of both Pro258 and Pro264 had altered folding kinetics and resulted in enzymes that expressed little catalytic activity. The Pro258-Pro258 bond is cis in its configuration, and the P258A mutant SHMT showed reduced thermal stability. Pro216, Pro258, and Pro264 are conserved in all 53 known sequences of this enzyme. The results are discussed in terms of the role of each Pro residue in maintaining the structure and function of SHMT and a possible role in pyridoxal 5′-phosphate addition to the apo-enzyme.

Serine hydroxymethyltransferase (SHMT) catalyzes the reversible interconversion of serine and glycine with the one-carbon group being transferred to H₄PteGlu as the 5,10-CH₂ adduct (1). This reaction is the major source of one-carbon groups in the cell that are required for thymidylate, purine, and methionine biosynthesis (2). SHMT is a member of the family of enzymes that contain tightly bound PLP as a cofactor. This family of enzymes is involved in many reactions in amino acid metabolism and in the biosynthesis of most neurotransmitters (3). When PLP is removed from these enzymes, they form the functionally inactive apo forms. Little is known about the in vivo mechanism of activation of the apo forms of B6 enzymes by PLP addition. Here we continue our study on understanding the mechanism of PLP addition and its relationship to the folding mechanism of eSHMT.

How and when PLP is added to apo-eSHMT from Escherichia coli during its biosynthesis and folding has been previously addressed (4–6). Folding studies show that two domains fold rapidly and form a dimer without the involvement of PLP. An N-terminal segment of approximately 55 residues and an interdomain segment of approximately 50 amino acids remain sensitive to protease digestion until the final rate determining step in the formation of native apo-eSHMT. This 50-amino acid segment between the two domains not only contains Lys229, which forms the internal aldimine bond with the 4′-aldehyde of PLP, but several other residues involved in PLP binding. PLP appears to bind only to the fully formed apo enzyme. Denaturation studies in urea show that release of PLP from the active site is one of the first events to occur in the unfolding process, which is in agreement with the addition of PLP during or after the final folding step. The final folding step is very temperature-sensitive, with a half-life of 40 min at 4 °C and approximately 1 min at 30 °C. The rate determining step exhibits an enthalpy of activation of 22 kcal mol⁻¹ (6). At the boundaries of the interdomain segment are 5 Pro residues: 3 at the N-terminal end and 2 at the C-terminal end. The enthalpy of activation of converting a trans X–Pro peptide bond to the cis configuration is also approximately 20 kcal mol⁻¹ (7). We proposed that one or more of these Pro residues would be in the cis configuration (6). Since those folding studies were published, the three-dimensional structure of eSHMT has been determined, and 1 of the 5 Pro residues is in the cis configuration. In this study we report the effect of changing each of the 5 Pro residues that flank the interdomain stretch of 50 amino acids to both Ala and Gly and determine the effects of these mutations on catalytic activity, stability, and folding kinetics. We conclude that 2 of the Pro residues are not critical for the folding process but that the other 3 Pro residues are important in folding and maintaining catalytic activity. The Pro that is most critical is the one that is in the cis configuration. From the three-dimensional structure of the enzyme, we discuss the role of the 3 critical Pro residues in maintaining a competent active site. We also discuss the possible role of this 50-residue interdomain segment in the mechanism of converting apo-eSHMT into holo-eSHMT.

EXPERIMENTAL PROCEDURES

Materials—All coenzymes, buffers, amino acids, column chromatography supports, reagents for bacterial growth, and ultra pure urea were purchased from either Sigma-Aldrich or Fisher. Methyleneetetrahydrofolate dehydrogenase was purified from an E. coli clone as previously described (8). Oligonucleotide primers for making mutant forms of eSHMT were obtained from Invitrogen. Mutant forms of eSHMT were made with the Transformer site-directed mutagenesis kit from Clon-
Serine Hydroxymethyltransferase

Expression, Purification, and Activity of Pro Mutants of eSHMT—As reported previously the 3 Trp residues Trp<sup>16</sup>, Trp<sup>183</sup>, and Trp<sup>385</sup> in eSHMT can be replaced with Phe residues without significant alterations in the catalytic and stability properties of the enzyme (5). At the time when this work was published a three-dimensional structure of the E. coli enzyme was not available. From studies using FRET measurements of site mutants, proteolytic digestion studies during folding and fluorescence quenching of Trp residues, we proposed a detailed mechanism for the folding of eSHMT (5, 6). A model of the final folding intermediate involved in the rate determining conversion to apo-eSHMT is shown in Fig. 1. Proteolytic digestion studies during folding showed that the active site Lys<sup>229</sup>, which forms the aldimine with PLP, is present on a sequence of approximately 50 amino acids between two rapidly folding domains that were protease resistant (Fig. 1, Domains 1 and 2). In addition to this interdomain segment, the N-terminal 55 amino acid residues were also susceptible to protease digestion, suggesting that this region of the protein was not also in its native state. The protease-resistant domain 1 stretches from Tyr<sup>55</sup> to Thr<sup>224</sup>, and domain 2 stretches from Leu<sup>276</sup> to the C-terminal Ala<sup>417</sup>. The rate determining step in forming the fully protease-resistant enzyme was the “folding in” of the N-terminal 55 amino acids and the interdomain segment, which occur at the same rate. We noted that the boundaries of the interdomain segment are populated by 5 Pro residues (Fig. 1). Pro residues 214, 216, and 218 are near the C terminus of domain 1, and Pro residues 258 and 264 are in the interdomain segment near the N terminus of domain 2. To probe the function of these Pro residues on the folding mechanism of eSHMT, each was changed to an Ala and Gly residue. The mutations for Pro<sup>114</sup>, Pro<sup>148</sup>, and Pro<sup>168</sup> were done in the protein containing only the Trp at position 183, which is referred to as W183 eSHMT. The mutations of Pro<sup>258</sup> and Pro<sup>264</sup> were done in the construct containing only Trp<sup>385</sup>, which is eSHMT was reduced with NaCNBH<sub>3</sub> as previously described (6). The PLP aldimine bond of the holoenzyme is reduced by this procedure to a stable secondary amine of PyP and is referred to as eSHMT-PyP. Reduction occurs with a spectral shift from 422 nm to 335 nm. The rate determining step in the folding and fluorescence quenching of Trp residues, we proposed a detailed mechanism for the folding of eSHMT (5, 6). A model of the final folding intermediate involved in the rate determining conversion to apo-eSHMT is shown in Fig. 1. Proteolytic digestion studies during folding showed that the active site Lys<sup>229</sup>, which forms the aldimine with PLP, is present on a sequence of approximately 50 amino acids between two rapidly folding domains that were protease resistant (Fig. 1, Domains 1 and 2). In addition to this interdomain segment, the N-terminal 55 amino acid residues were also susceptible to protease digestion, suggesting that this region of the protein was not also in its native state. The protease-resistant domain 1 stretches from Tyr<sup>55</sup> to Thr<sup>224</sup>, and domain 2 stretches from Leu<sup>276</sup> to the C-terminal Ala<sup>417</sup>. The rate determining step in forming the fully protease-resistant enzyme was the “folding in” of the N-terminal 55 amino acids and the interdomain segment, which occur at the same rate. We noted that the boundaries of the interdomain segment are populated by 5 Pro residues (Fig. 1). Pro residues 214, 216, and 218 are near the C terminus of domain 1, and Pro residues 258 and 264 are in the interdomain segment near the N terminus of domain 2. To probe the function of these Pro residues on the folding mechanism of eSHMT, each was changed to an Ala and Gly residue. The mutations for Pro<sup>114</sup>, Pro<sup>148</sup>, and Pro<sup>168</sup> were done in the protein containing only the Trp at position 183, which is referred to as W183 eSHMT. The mutations of Pro<sup>258</sup> and Pro<sup>264</sup> were done in the construct containing only Trp<sup>385</sup>, which is
referred to as W385 eSHMT. Having only one Trp residue permits an examination of the effect of these Pro residues on the folding of the domain with which they are most closely associated without interference of the fluorescence properties of the other two Trp residues.

Each mutant enzyme was tested for catalytic activity and affinity for serine and H4PteGlu (Table I). Only P216G, P258A, P258G, P264A, and P264G exhibited greatly reduced catalytic activity. With P258A and P258G the activity is so low that Km never, with P258A the addition of serine caused a spectral shift of the bound PLP from 406 to 416 nm. This spectral shift permitted the determination of a Kd for l-serine of 8.0 mM. The Kd value of 8 mM is 25-fold higher than the Kd value for l-serine with the wild-type enzyme. The other Pro mutants exhibited Kd values for serine that are not significantly different from wild-type eSHMT. Only P216G showed a significant change in the affinity for H4PteGlu, which is 5.5-fold higher than wild-type eSHMT.

Spectral Properties—With the exception of P258A and P258G, the spectral properties of each mutant eSHMT exhibits absorbance maxima at 418 and 322 nm. The wild-type enzyme shows a single absorbance maxima at 422 nm. The 322-nm peak was approximately 30–50% of the height of the 418-nm peak and varied with the mutant and the age of the enzyme preparation. This suggests that some of the bond PLP is not in its catalytically active form. This probably accounts for many of the Kd values being only approximately 200 instead of 300 under our assay conditions. The importance of these spectral differences will be addressed under “Discussion.”

Each mutant protein was analyzed for secondary structure using circular dichroism spectroscopy. There was no significant difference in circular dichroic spectra between the mutant and the wild-type enzymes. Also, we had previously shown by circular dichroism that apparently complete secondary structure forms in less than a minute at 4 °C, but the final folding step takes up to 15 h to complete at this temperature (6). We checked the circular dichroic spectrum of wild-type enzyme at 4 °C during the first 10 min after refolding had been initiated by dilution of an 8 M urea unfolded solution. The sample was then warmed to 30 °C, at which folding is completed in a few minutes. A second scan at this temperature showed no significant difference from the original spectrum taken at 4 °C. We conclude that the secondary structural elements are essentially complete after a few minutes at 4 °C when the enzyme is represented as the structure shown in Fig. 1.

Stability of Pro Mutants—The ability of each mutant to undergo a reversible unfolding-folding cycle in increasing concentrations of urea was determined (5). A plot of fluorescence emission at 330 nm versus urea concentration showed a sigmoidal decrease in fluorescence with increasing concentration of urea characteristic of a two-state folding curve. However, because each mutant enzyme contained only Trp83 or Trp85, this method follows only the degree of folding of either domain 1 or domain 2. The midpoint of the curve for W183 eSHMT is 2.5 M urea. Mutants P214A, P214G, and P218 A also had midpoints at 2.5 M urea. Mutants P216A and P216G exhibited midpoints at 2.0–2.2 M urea, showing that domain 1 in these mutants is slightly less stable. The urea midpoint of W385 eSHMT is 3.0 M urea (6). The midpoint for P258A and P258G were also at 3.0 M urea, but for P264A and P264G the midpoint was at 2.5 M. These results suggest that the mutation of the 5 proline residues has only marginal effects on stability of domains 1 and 2 as determined by unfolding in urea.

The thermal stability of each alanine mutant protein was determined by differential scanning calorimetry. The thermal denaturation of eSHMT is irreversible. An apparent Tm (Tm appellant) was determined from the peak in the thermogram for each Pro mutant in the absence and presence of 30 mM l-serine (Table II). Each Pro mutant showed similar heat stability Tm (Tm appellant = 63–67 °C) and stabilization of approximately 5 °C upon the addition of serine. The exception is the P258A mutant, which exhibits a double thermal transition that is considerably lower than wild-type enzyme (Fig. 2). For this mutant there is no increase in thermal stability upon binding serine, even though spectral studies showed that serine binds to the active site with a Kd of 8 mM (Table I). Most of the denaturation traces exhibited nonsymmetrical curves. This has been shown before to result from different domains in the enzyme unfolding at different temperatures and is consistent with the urea denaturation curves showing that domain 2 is slightly more sta-

### Table I
Comparison of kinetic and folding properties of pro mutants of eSHMT

| Mutant | Km (Ser) | Km (H4PteGlu) | kcat | kfold 1<sup>a</sup> | kfold 2<sup>a</sup> |
|--------|----------|--------------|------|--------------------|--------------------|
| Wild type | 0.30 | 15 | 300 | 0.011 (100)<sup>b</sup> | 0.0082 (90) |
| P214A | 0.14 | 20 | 200 | 0.104 (10) | 0.0065 (71) |
| P214G | 0.24 | 20 | 200 | 0.042 (29) | 0.0032 (34) |
| P216A | 0.25 | 20 | 200 | 0.028 (66) | 0.0032 (34) |
| P216G | 0.58 | 85 | 36 | No FRET signal | 0.0065 (48) |
| P218A | 0.13 | 19 | 270 | 0.056 (52) | 0.0074 (62) |
| P218G | 0.13 | 17 | 200 | 0.059 (38) | 0.0036 (59) |
| P258A | 8.0 | 0.5 | No FRET signal | 0.0051 (90) |
| P258G | 0.33 | 17 | 98 | 0.016 (41) | 0.0051 (90) |
| P264A | 0.33 | 17 | 98 | 0.016 (41) | 0.0051 (90) |
| P264G | 1.33 | 7 | 0.059 (10) | 0.0051 (90) |

<sup>a</sup>Rate constant for the appearance of fluorescence emission at 384 nm caused by fluorescence energy transfer.

<sup>b</sup>The percentage of the signal amplitude that is associated with the given rate constant.

### Table II
Thermal stability of SHMT Pro mutants as determined by differential scanning calorimetry

| SHMT | Tm<sub>app</sub> −Ser | Tm<sub>app</sub> +Ser | Tm<sub>app</sub> |
|------|----------------|----------------|-------------|
| W183 | 66.5 | 73 | 6.5 |
| apoW183 | 63 | 69 | 6 |
| P214A | 70 | 73.5 | 3.5 |
| P216A | 66 | 71 | 5 |
| P258A | 53.5 (60.5)<sup>a</sup> | 51 (60.5) | 0 |
| P264A | 57.5 | 61.5 | 4 |

<sup>a</sup>This represents the highest molar heat capacity value and is not exactly equal to a defined Tm value because the denaturation process involves irreversible aggregation. It is defined here as an apparent Tm (Tm<sub>app</sub>).
ble than domain 1 (12). Apo-W183 eSHMT denatures approximately 3–4 degrees lower than holo-W183, suggesting that PLP binding does not greatly increase thermal stability in eSHMT (Table II).

Kinetics of Refolding—The refolding rate was determined by using FRET, which occurs during the final rate determining step in folding when the interdomain segment and the N-terminal 55 residues collapse into the native structure. The fluorescence traces for P214A and P216A eSHMT are shown in Fig. 3. We had previously shown that this final step in folding follows a first order reaction (6). In these studies of Pro mutants, the traces are best fit by two parallel first order reactions. The two first order rate constants and their relative associated amplitudes (shown in parentheses) are recorded in Table I. The slower of the two rate constants is in the 0.008 s$^{-1}$ range. These values are close to the values observed for wild-type eSHMT under these same conditions. The faster rate is not observed with wild-type eSHMT. Note that in the refolding of P214A, 90% of the amplitude follows a first order refolding path. With P216A 66% folds by the faster rate, and the 34% folding at the slower rate occurs approximately 2-fold slower than P214A. P216G, P258A, and P258G give no FRET signal, suggesting that in the final structure the PyP group is more than 40 Å distant from the Trp residue associated with its closest domain.

Three-dimensional Structure of eSHMT—Fig. 4 shows the location of each of the 5 Pro residues, with respect to the active site PLP (shown in green). Pro$^{214}$, Pro$^{216}$, and Pro$^{218}$ are part of a structural motif from Ala$^{210}$ through His$^{201}$ that forms a long loop between a β strand (Val$^{222}$–Thr$^{228}$) and an α helix (Ala$^{215}$–Ala$^{209}$). Although the loop is exposed to the solvent, the β strand and the α helix are buried and form a part of the
catalytic site. Some of the residues from the β strand and α helix and others immediately preceding or following these secondary structures are known to play significant roles in PLP binding and catalytic activity. These include Asp200, His203, His228, and Lys229 (13). Interestingly, Pro216 is located in the middle of the loop, and whereas the side chains for Pro214 and Pro218 face the bulk solvent, that of Pro216 faces the interior of the protein and forms part of a hydrophobic patch. The Pro258-Pro264 motif is also a loop, with the two Pro residues located at the ends of the loop and initiating and terminating two α-helices (Glu246, Phe257 and Leu265, Glu278, respectively). The loop region and part of the α-helices form a significant part of the catalytic site. The amide nitrogen of Gly263 makes a short hydrogen bond interaction with the phosphate of the PLP in the other monomer. The Pro258-Glu259, Gly260-Gly261-Gly262-Gly263-Pro264 motif is located at the interface of the two subunits and is involved in many direct hydrogen bond interactions across the subunit interface, including Asn134ND2 to Pro258O, Asn134ND2 to Gly259O, Phe135N to Gly259O, Glu100NE2 to Gly260O, and Ser99OG to Gly262N. There are a number of significant water-mediated interactions involving the loop region and residues from the other subunit and with PLP (Fig. 5).

**DISCUSSION**

**Pro Residues and Folding**—The role of Pro residues in protein folding has been widely discussed and studied (14, 15). Pro residues often act to slow down the folding process creating intermediates on the folding pathway. Of particular interest is the role of cis Pro residues that often cap α-helices or are involved in β turns. The reversible cis-trans interconversion of X-Pro peptide bonds exhibits a large enthalpy of activation of approximately 20 kcal mol\(^{-1}\) (15). In the unfolded state the equilibrium favors the more stable trans form by a 3 or 4 to 1 ratio. Most Pro residues in proteins are trans, but approximately 6% are cis (16). In small proteins and some larger ones Pro isomerization can be the rate determining step in folding and because Pro residues exist as a mixture of isomers in the unfolded state leads to multiple parallel pathways in folding (15, 17). In trying to discern the role of Pro residues in the folding of larger multidomain proteins, it is helpful to have structural information about intermediates in which Pro residues play a role.

**Structure of the Folding Intermediate in eSHMT**—The folding mechanism of eSHMT has been elucidated previously, and by using a variety of methods we were able to define several steps and intermediates on the reversible folding-unfolding pathway (4–6). For our purposes in this study we divide the folding mechanism into two phases: a relatively rapid phase lasting less than a minute and involving several intermediates in which two domains have folded into their native state and a dimer is formed and a slow final phase that involves the folding in of the N-terminal 55 amino acid residues and a 50-residue interdomain segment. The final rate determining step is described by a single first order rate as determined by using information from FRET studies between three widely spaced Trp residues and an active site PyP, suggesting that the first rapid steps in folding lead to a single structural intermediate. The slower final rate determining step of this multidomain homodimer is complete in approximately 5 min at 37 °C and exhibits an energy of activation of 22 kcal mol\(^{-1}\), leading to the prediction that the formation of a cis X-Pro peptide bond is involved.

Subsequent to these folding studies the three-dimensional structure of several SHMT proteins, including eSHMT, were determined (13, 18–21). The SHMT structures from human, mouse, rabbit, *E. coli*, and *Bacillus stearothermophilus* all have a cis X–Pro bond equivalent to Pro258 in eSHMT. These struc-
The Pro residues, PLP, and 5-formyl H$_2$PteGlu (listed as FFO) are shown in black, blue, and red space filling models, respectively. A, the enzyme with the interdomain segment (Val212–Ala279) lifted out of the folded enzyme. B, enzyme with the interdomain segment located in the fully folded enzyme.

The Size of Domains 1 and 2—The intermediate exhibits putative hinge points. The positions of all 5 Pro residues that precede this residue. Domain 2 most likely starts at a small loop region from Ala$^{279}$ to Glu$^{283}$. The subtilisin site at Lue$^{276}$ is part of a helix and an unlikely hinge point. These predictions would then define the structure of the intermediate as the N-terminal segment from residue 1 to Pro$^{60}$, domain 1 from Lys$^{82}$ to Ala$^{210}$, the interdomain segment from Val$^{212}$ to Ala$^{279}$, and domain 2 from Phe$^{284}$ to Ala$^{317}$. Pascarella et al. (22) have defined a special algorithm for comparing sequence alignments of SHMT proteins. Using their alignments in 53 SHMT structures from a wide variety of organisms, we found that our putative first hinge point Gly$^{61}$ is conserved in 39 of 53 known structures of SHMT and its preceding Pro$^{60}$ is conserved in 49 of the 53 structures. The second predicted hinge point at Gly$^{211}$ is conserved in 45 of 53 SHMT structures, and those that do not contain Gly$^{211}$ have either a preceding Gly or Ala residue. The small loop structure at Ala$^{279}$–Glu$^{283}$ is also highly conserved with 43 sequences having an equivalent Ala$^{212}$ and 42 sequences having either a Glu$^{283}$ or Asp$^{283}$. Also, more than half of the sequences have a conserved Pro$^{282}$ that precedes the Glu$^{283}$ found in eSHMT. These putative hinge points will be tested in future studies.

Secondary Structure of the "Intermediate"—Fluorescence polarization studies showed that the PyP group attached to the ε-aminogroup of Lys$^{229}$ was rotating freely (6). However, we could make no statement about the secondary structural aspects of the N-terminal segment or the interdomain segment in our model shown in Fig. 1. We now know that both of these segments contain considerable α-helices and β-strands. We calculate that 28% of the α-helices and 25% of the β-strands are located in the N terminus and interdomain segments. However, the circular dichroic spectrum of the enzyme taken at 4 °C after initiation of refolding does not change by more than 2 or 3% from the spectrum of the fully folded enzyme. Therefore, we conclude that the N-terminal segment and the interdomain segment contain most, if not all, of the secondary structural elements found in the fully folded enzyme.
Role of Pro$^{214}$ and Pro$^{218}$—Changing Pro$^{214}$ and Pro$^{218}$ to Ala or Gly has little effect on the catalytic activity or thermal stability of the enzyme. The decrease in activity is most likely related to the folded form where the PLP absorbs at 322 nm. The affinity for both 1-serine and H$_2$PteGlu remain largely unchanged for these two Pro mutants, suggesting that the active site of the enzyme is not greatly changed. The fact that both Pro$^{214}$ and Pro$^{218}$ are located on the surface of the enzyme suggests that these residues do not play a critical role in the final folding step. An examination of 53 sequences of SHMT from a wide spectrum of organisms shows that 36 retain a Pro equivalent to Pro$^{214}$ in eSHMT. Likewise, 23 SHMT sequences retain a Pro residue equivalent to Pro$^{218}$ in eSHMT. We conclude that although Pro$^{214}$ and Pro$^{218}$ may play some role in the final folding step, it is not a crucial role.

Role of Pro$^{216}$—The P216A mutant exhibits catalytic and stability properties that resemble those of the wild-type enzyme. However, the P216G mutant has very low activity and could only be purified in poor yield. Pro$^{216}$ lies in the middle of a loop and points to the interior of the protein and is a part of a hydrophobic cluster that includes Phe$^{106}$, Trp$^{103}$, Val$^{204}$, Tyr$^{213}$, Val$^{199}$, Met$^{201}$, Val$^{208}$, and Val$^{223}$. Apparently substitution by an Ala maintains this hydrophobic patch, but substitution with Gly results in the loss of its integrity, leading to serious misfolding problems. Of interest is that all 53 SHMT sequences have a Pro at this position.

Roles of Pro$^{258}$ and Pro$^{264}$—The most critical residues examined in this study are Pro$^{258}$ and Pro$^{264}$. Pro$^{258}$ is in a cis configuration in the five known structures from mouse, human, rabbit, E. coli, and B. stearothermophilus. Our evidence that the secondary structure of the interdomain segment has already been formed in the intermediate would suggest that this Phe-Pro bond is already cis and would explain why the final folding step occurs as a single exponential reaction in wild-type eSHMT. Both Pro$^{258}$ and Pro$^{264}$ lie next to the PLP ring in the adjoining subunit (Fig. 5). Any changes in the orientation of these two residues can alter the orientation of the PLP ring. During a catalytic cycle the PLP rotates to accommodate the incoming amine substrate and the leaving of the PLP aldimine bond is on the backside of the structure as it is shown. In Fig. 6A the PLP is exposed, but in 6B it is clear that in the final folded structure the PLP is buried. The figure shows, however, that 5-CHO-H$_2$PteGlu is bound largely at the surface of the enzyme.

We propose that the final folding step is involved in PLP binding. Removing the 50-residue segment (shown in Fig. 6A) exposes the PLP site to solvent. Our hypothesis is that in the absence of PLP the interdomain and N-terminal segments are in equilibrium with a more open structure that is similar to the intermediate shown in Fig. 1. In this more open structure PLP can bind at a pre-site and then is folded in to form the active site. The new contacts between the coenzyme and the protein stabilize this closed form, resulting in the tight binding properties seen in all PLP enzymes. Our previous studies showed that in denaturation at low urea concentrations, the first event is the release of PLP into solvent but that this event did not greatly alter the denaturation properties of domains 1 and 2. This is confirmed in this study where the thermal denaturation of apo-W183 is very similar to that of holo-W183 (Table II). When the PLP aldimine bond is reduced with NaCNBH$_3$, it now requires a much higher concentration of urea (midpoint is 6 M urea) and temperature ($T_m$ of 82.4 °C), because the first step in the unfolding pathway has been greatly stabilized (6).

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