The Conserved N-capping Box in the Hydrophobic Core of Glutathione S-Transferase P1-1 Is Essential for Refolding

IDENTIFICATION OF A BURIED AND CONSERVED HYDROGEN BOND IMPORTANT FOR PROTEIN STABILITY

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Glutathione S-transferases (GSTs; EC.2.5.1.18) are a family of detoxification enzymes that catalyze the nucleophilic attack of glutathione (GSH) on the electrophilic groups of a wide range of hydrophobic compounds (1–4), including herbicides, insecticides, carcinogens, and other xenobiotic substances (5–7). The cytosolic GSTs have been grouped into five evolutionary classes, Alpha, Pi, Mu, Theta, and Sigma on the basis of N-terminal sequences, substrate specificity, and immunological properties (8–9). The GSTs are dimeric proteins (molecular mass about 50 kDa) assembled from identical or non identical subunits from the same gene class.

Although the alignment of all known GST sequences shows that only 6–7 amino acid residues are strictly conserved, the representative crystal structure of each cytosolic class shows that the overall polypeptide fold is very similar (10–13). Each subunit is characterized by two distinct domains and possesses an active site that acts independently of the other subunit. The smaller N-terminal domain (domain I) adopts an α/β topology and contributes most of the contacts to GSH. The C-terminal domain (domain II) is an all helical structure and provides some of the contacts to the hydrophobic binding site that lies adjacent to the GSH binding site. A recent study (14) has also shown that all GSTs and GST-related proteins, including several uncharacterized proteins with significant sequence similarity to GSTs, are characterized by the presence of two conserved sequence motifs. Motif I is found in the first domain and consists of a sequence encompassing β-sheets β4, β5 and the α3-helix. Motif II is in the C-terminal domain and includes a long, conserved loop and the subsequent α3-helix. Gly-146 and Asp-153, belonging to this latter motif, represent the only two residues that are strictly conserved in GST domain II. The role of Gly-146 is still unknown. Some authors have previously attempted to determine the role of Asp-153 through site-directed mutagenesis, but the results are somewhat contradictory (15–16).

We have recently shown (17) that Asp-153 has a structural role not by itself but as being part of the motif (Ser/Thr-Xaa-Xaa-Asp) named “capping box” (18–20), which is present at the beginning of the α6-helix. The capping box is essential for refolding of the denatured protein at a physiological temperature. The results suggest that during folding this buried and conserved motif, making a definite set of native-like contacts, determines the formation of a specific folding nucleus that probably represents a transition state of the folding process.

The second domain of cytosolic glutathione S-transferases (GSTs) contains a strictly conserved N-capping box motif (Ser/Thr-Xaa-Xaa-Asp) at the beginning of α6-helix in the hydrophobic core of the molecule. Considering the specific function attributed to capping box residues in the helix nucleation, we decided to investigate, by site-directed mutagenesis, the role that this motif could have in the folding and stability of human GSTP1-1. Both capping box mutants, S150A and D153A, were significantly more thermostable than wild-type GSTP1-1, indicating that the local destabilization of the α6-helix determined by a single capping residue mutation affects the overall stability of the protein. The results also show that, in addition to capping interactions, an important role in the stability of the final structure of the protein is played by a buried and conserved hydrogen bond formed between the side chain of Asp-153 and the amide NH of Ile-144 located in the long loop preceding α6-helix. Reactivation experiments in vitro indicate that the N-capping box is essential for refolding of the denatured protein at a physiological temperature. The results suggest that during folding this buried and conserved motif, making a definite set of native-like contacts, determines the formation of a specific folding nucleus that probably represents a transition state of the folding process.

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The abbreviations used are: GST, glutathione S-transferase; GSH, glutathione; CDNB, 1-chloro-2,4-dinitrobenzene; GdnHCl, guanidine hydrochloride; DTT, dithiothreitol; Ncap, first residue of the N-capping box motif located at the beginning of an α-helix.
plays a key role during refolding as well as in the final structure of the protein.

EXPERIMENTAL PROCEDURES

Materials—Wild-type human GSTP1–1 was obtained by expression of a cloned cDNA in *Escherichia coli* XL-1 blue (Stratagene, La Jolla, CA) as described previously (21). GSH and 1-chloro-2,4-dinitrobenzene (CDNB) were purchased from Sigma.

Preparation and Assays of Mutant Enzymes—The oligonucleotide primer sequences used for site-directed mutagenesis were as follows: for the S150A mutation, 5'-CAT ATG GCT TTC GCC GAT TAC AAC CT-3'; for the D153A mutation, 5'-CAT ATC TCC TTC GCT GCC TAC AAC CT-3'; and for the S150A/D153A mutation, 5'-CAT ATG GCC GCC TAC AAC CT-3'. The oligonucleotides were phosphorylated before use and were then used in pairs with one mutant primer and the reverse primer in each mutagenesis reaction. The expression construct pKHP1 (21) encoding human GSTP1–1 was used as template DNA.

The reaction mixture contained 0.8 μM of each primer, 0.2 mM dNTP mix, varying amounts of template DNA, 2.5 units of *Pfu* DNA polymerase (Stratagene) and the buffer supplied with the DNA polymerase in a total volume of 50 μL. After a hot start at 94 °C for 10 min, *Pfu* DNA polymerase was added, and 25 cycles of mutagenesis/amplification followed: 94 °C for 1 min, 68–70 °C for 1 min, and 72 °C for 7 min. The program was ended at 30 min at 72 °C.

The PCR product was recovered from agarose gel after electrophoresis and was ligated before transformation of *E. coli* XL-1 blue. Small scale expression tests were performed and high level-expressing clones, based on SDS-polyacrylamide gel electrophoresis using Phastsystem (Pharmacia, Uppsala, Sweden), were chosen for DNA sequence analysis (22). Mutant clones were then used for large scale expression of protein.

Protein Expression and Stability in Vivo as a Function of the Growth Temperature of the Host Cell—Cultures of *E. coli* XL-1 blue containing plasmids were grown in 500 ml of expression medium in a 2-liter Erlenmeyer flask at 37 °C. At an *A* 

Temperature Dependence of Refolding in Vitro for Wild-type and Capping Mutants—When the refolding of human GSTP1–1 and its mutants was to be monitored, 10 μM enzyme was first denatured in 4 mM GdnHCl (0.2 mM phosphate, 1 mM EDTA, 5 mM DTT, pH 7) at 25, 32, and 40 °C for 30 min and then diluted (defining time 0) 1:40 into renaturation buffer (0.2 mM phosphate, 1 mM EDTA, 5 mM DTT (pH 7)) at the same temperature. The final GdnHCl concentration was 0.1 mM during refolding. All refolding dilutions were carried out by rapid addition of the renaturation buffer to denatured enzyme. Activity recovery was monitored by withdrawal at time *t* of appropriate aliquots of the renaturation mixture immediately diluted into 2.0 ml of assay buffer.

Molecular Graphics Analysis—Coordinates of GST x-ray structures were derived from the Brookhaven National Laboratory Protein Data Bank via anonymous file transfer protocol. The crystal structures were rearranged (Hyperchem program; Autodesck, Sausalito, CA) by energy-gradient minimization with an Amber3-based Polak-Ribiere conjugate gradient minimization algorithm (25). Rearrangements were stopped when a gradient of less than 0.2 kcal/mol per Å was reached.

RESULTS

Expression and Purification of Capping Box Mutant of Human GSTP1–1—To investigate the role of the conserved capping box residues, Asp-153 and Ser-150 in human GSTP1–1 were individually replaced with alanine producing D153A, S150A, and S150A/D153A mutants by oligonucleotide-directed mutagenesis. Mutant and wild-type enzymes were expressed in *E. coli* and purified by affinity chromatography on immobilized GSH. The purified proteins gave a single band on SDS-polyacrylamide gel electrophoresis (not shown). In each case, the unbound activity fraction was very low (2–5% of the total activity, not shown), suggesting that the affinity of mutant enzymes for GSH-Sepharose column was essentially the same as that of the wild type. Considering that the above substitutions could represent temperature-sensitive mutations, protein expression was performed at different temperature of host cell growth. The intracellular yields of the S150A mutant and wild-type GSTP1–1 were similar and essentially independent of the temperature of culture medium in the range of 25–40 °C (not shown). On the contrary, the yields of D153A and S150A/
D153A mutants were lower and clearly decreased with increasing temperature. This probably reflects the fact that D150A and S150A/D153A molecules fail to reach the native state at a physiological temperature. In fact, the cytosolic-specific activities as well as the specific activities of the purified D150A and S147A/D150A mutants sharply decreased with increasing temperature of cell growth (not shown).

Kinetic and Structural Properties of Wild-type and Capping Mutant Enzymes Expressed at 25 °C—

Table I summarizes the kinetic parameters of the CDNB conjugation catalyzed by the wild-type GSTP1–1 and capping mutants. The replacement of Ser-150 with alanine does not significantly affect the kinetic parameters as compared with the parent enzyme. On the contrary, the D153A mutant as well as S150A/D153A double mutant showed limited but significant kinetic differences when compared with wild-type. In particular, while the $K_m$ values were substantially unaffected by the mutations, the $k_{cat}$ and then the $k_{cat}/K_m$ for the D153A and S150A/D153A mutants were about 1.5-fold higher than those of parent GSTP1–1.

When wild-type GSTP1–1 and its mutants were expressed at permissive temperature (25 °C), their structural properties were very similar. The far-UV CD spectra (Fig. 1) as well as their gel filtration retention times (not shown) were the same, suggesting that the capping mutants and wild-type enzyme, in terms of secondary structure and dimeric arrangement of the molecule, were essentially identical. Table II shows the intrinsic fluorescence properties of these proteins. The $\lambda_{max}$ was the same, indicating that a similar polarity characterizes the tryptophanyl environments of all enzyme variants. The fact that the normalized intensities of fluorescence were significantly different suggests that only limited conformational changes affect mutants when compared with the wild-type enzyme. These minor differences involve tertiary contacts of tryptophanyl residues located in the GST domain I, far from the mutation sites.

Thermal Stability of the Wild-type and Mutant Enzymes—

Heat inactivation of wild-type and capping mutants, expressed at permissive temperature, was investigated. The enzymes were incubated in 0.01 M potassium phosphate (pH 7.0) at various temperatures for 10 min, and the remaining activities were assayed in 0.1 M potassium phosphate (pH 6.5) at 25 °C (Fig. 2). The results indicate that single capping residue substitutions cause very large effects on the catalytic competence even though the active site is situated far from the location of the mutations. In particular, the S150A mutant was more...
unstable than the wild-type enzyme, being almost completely inactivated at 50 °C. In contrast, the wild-type, under the same conditions, was inactivated to less than 10%. It should be noted, however, that the S150A mutant, at physiological temperature (37–40 °C), was stable for several hours (not shown). On the other hand, the D153A and S150A/D153A mutants were much more unstable than the wild-type GSTP1–1 and the S150A mutant, being completely inactivated by incubation at 40 °C for 10 min. For these last two mutants, some inactivation was observed even at a temperature as low as 32 °C. Time courses of thermal denaturation (not shown) demonstrated that after 30 min of incubation at 32 °C both the D153A and S150A/D153A mutants were inactivated by about 30%. The slightly higher extent of inactivation shown by the D153A enzyme, at each temperature, compared with the S150A/D153A double mutant is explained by the occurrence of new interfering interactions due to the presence, in a nonpolar environment, of a serine residue without a complementary counterpart that could participate in hydrophobic bond formation.

**Reactivation Yields at Different Temperatures—**Considering the proposed role of the capping box motif in helical nucleation, single substitutions of N-capping residues could represent temperature-sensitive mutation affecting GST folding. To test this hypothesis, we have investigated the reactivation yields of GSTP1–1 and its mutants at different temperatures of refolding. 10 μM enzyme, expressed at 25 °C, was denatured in 4 M GdnHCl for 30 min. This denaturant concentration was sufficient to completely unfold the proteins as indicated by the loss of their CD signal at 222 nm (not shown). Successively, each unfolded enzyme was diluted 40-fold in the same denaturing tube with phosphate buffer at pH 7.0 to a final GdnHCl concentration of 0.1 M. Appropriate aliquots from this incubation mixture were immediately assayed for activity at 25 °C. A single exponential fitted all kinetic reactivation curves (not shown). In each case, 5–6 min of incubation in the refolding medium was sufficient to reach the maximum of reactivation, indicating that the refolding rates were very similar (Fig. 9). On the contrary, the reactivation yields of the capping mutants were very different from that of the wild-type protein with increasing temperatures of refolding. Fig. 3 shows in fact that, while the reactivation yield of wild-type enzyme was essentially unaffected by temperatures in the 25–37 °C range, the yields of mutants, even at a different extent, sharply fell with increasing temperature toward physiological values. At 25 °C, all enzyme variants showed the same recovery of specific activity. On the contrary, at 37 °C all mutants displayed very poor or complete lack of refolding activity even at the very beginning of the incubation. It should be noted that the S150A mutant, in distinguishing from the other two mutants, is stable at 37 °C. Thus the results indicate that the single Ncap S150A mutation destabilizes an essential intermediate of folding at 37 °C. The D153A and the S150A/D153A mutants showed much lower refolding yields even at 32 °C. It should be noted that most of the activity was regained in a few minutes, in which time period the inactivation of both mutants is substantially negligible. Thus, the results also suggest that the D153A mutation not only slightly affects the stability of the folded protein, but, to a higher extent, also destabilizes a productive intermediate of folding.

**Molecular Graphics Analysis—**The analysis of the three-dimensional structures of different GST indicates that the conserved sequence S/TXXD possess the typical properties of the capping box motif. Table III shows the reciprocal side chain-capping distances in the Pi, Mu, Alpha, Sigma, and *Schistosoma japonicum* GST structures. The side chain oxygen atom of the N3 Asp residue forms a hydrogen bond with the main chain NH of Ncap Ser/Thr (Table III, a), just as the side chain oxygen atom of Ncap Ser/Thr is within hydrogen bonding distance from the backbone NH of N3 Asp residue (Table III, b). Table III, c also shows that, in addition to capping interactions, Asp-153 in GSTP1–1 forms a very favorable hydrogen bond with the backbone NH of an hydrophobic residue (Ile 144 in GSTP1–1) located in the long loop preceding αc-helix (Fig. 4). It is interesting to note that this buried hydrogen bond, probably important in GST nucleus folding, is strictly conserved in the GST superfamily (Table III, c).

**DISCUSSION**

Understanding the pathway by which the native state of a protein is reached requires the identification of residues critical in the formation of kinetic intermediates and transition states of folding. In the present work, we have identified amino acid residues that influence stability and folding of GSTs and which are strictly conserved in this superfamily of proteins.

We have recently reported (17) that at the beginning of the GST αc-helix, in the hydrophobic core of the molecule, an N-capping box motif (Ser/Thr-Xaa-Xaa-Asp) that plays an important role in the helical propensity of the isolated αc-peptide is present. These results prompted us to investigate, by site-directed mutagenesis, the role that this conserved motif could have in the folding of protein.

To study folding and stability in vitro, GSTP1–1 and its corresponding capping mutants were expressed at 25 °C. At this permissive temperature, in fact, the kinetic parameters of all mutants were very similar to that of the wild-type enzyme. This similarity is in accordance with the data obtained by CD and fluorescence spectroscopy. The results show that secondary structures, dimeric arrangement, and the λmax of the intrinsic fluorescence were the same, indicating that the global folding of mutants and wild-type GST were similar. The fact that the

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**Table III**

Buried hydrogen bonds conserved at beginning of the αc-helix of the GSTs

| Alpha class | Mu class | Pi class | Sigma class | S. japonicum |
|-------------|----------|----------|-------------|-------------|
|             |          |          |             |             |
| a)          | (Asp)    | (Asp)    | (Asp)      | (Asp)       |
|             | OH···C−O | NH       | NH          | OH···C−O    |
|             | X        |          | Y           | Y           |
|             |          |          |             |             |
| b)          | NH       | OH       |             |              |
|             |          | (Asp)   |             |              |
|             |          | (X)      |             |              |
|             |          |          |             |              |
| c)          | OH···C−O |          |             |              |
|             | X        |          |             |              |
|             |          |          |             |              |

*a* Hydrogen bond degree.
The normalized intensity of fluorescence was significantly different suggests only limited differences in tertiary contacts surrounding one or both tryptophanyl residues of protein. However, all mutants showed a remarkable thermal inactivation when compared with the wild-type GSTP1–1 (Fig. 2). The loss of catalytic activity is explained by considering that the α6-helix, although located in the core of the protein, makes hydrophobic contacts with an important structural element of the active site (α1-helix of GST). Thus, single capping residue mutations that locally destabilize the conformation of the α6-helix also affect in a direct fashion the stability of the active site. Moreover, the N3 mutation (D153A) was much more destabilizing than the Ncap mutation (S150A). A simple explanation for this difference is given from the analysis of the three-dimensional structure of GST. As shown in Fig. 4, the conserved Asp-153 residue, in addition to capping-box interactions, forms a very favorable hydrogen bond with the amide NH of Ile-144. Moreover, this specific interaction, which is important for stabilization of the long loop preceding the α6-helix (GST motif II), is strictly conserved in GST classes (Table III).
Thus the results indicate that, in addition to capping-box interactions, an important role in the stability of the final structure of GST is played by this buried and conserved hydrogen bond. This also means that the appropriate conformation of GST motif II (14) is crucial in the free energy minimization of the final state and well compensates the energetic penalty imposed by the desolvation of a carboxylic group located in the interior of the protein with a low dielectric constant.

Refolding in vitro suggests that thermal lability of the final structure of mutants reflects differences in the conformational properties of a productive intermediate of folding. Reactivation in vitro of all mutants, in fact, was very thermosensitive. In particular, although the final structure of S150A mutant is stable at 37 °C, the yield of the refolded protein at this temperature is low. This indicates that S150A represents a temperature-sensitive mutation that identifies a residue critical for folding. Since Ser-150 in GSTP1–1 corresponds to an Ncap residue (18–20), this means that the N-capping box, highly conserved in the hydrophobic core of GSTs, is essential for refolding at physiological temperature. In addition to the specific role of governing helix propensity previously attributed (17) to this motif by studies of the isolated α-helical peptide, the present results also indicate that the helical conformation of this polypeptide segment is an important feature of a productive intermediate of folding. The results obtained by the peptide approach, as well as the fact that the S150A mutant is able to refold at a more permissive temperature (25 °C), further support this finding. We have previously observed (17) that the helical propensity of the isolated peptide sharply decreased by increasing the temperature. While at lower temperature, other determinants also contribute to helix formation; at 37 °C, the isolated α-helical peptide showed a significant helical content only in the presence of the capping box motif.

Much lower refolding yields were observed for the D153A and S150A/D153A double mutants than for the S150A mutant. It is conceivable that, in addition to capping-box interactions, an important role in the refolding could be played by the specific Ile-144—Asp-153 hydrogen bond identified in the crystal structure of the native enzyme described above. The results seem to indicate that, in addition to the α-helical structure, the appropriate package of this polypeptide segment to the preceding long loop is required for the formation of a productive folding intermediate. The conserved Ile-144—Asp-153 interaction could favor just one of several closely related conformations occurring during this hydrophobic packing and then it could help to establish GST conformational specificity. At the beginning of the α-helix, the presence of a conserved hydrophobic-staple motif (17), flanking the N-capping box, further supports this behavior. It has recently been observed (26–27) that such local sequences, when simultaneously present at the N terminus of a helix, have an important role in protein folding by determining the direction of the forming helix with respect to the preceding structural element. In addition, it should be noted that this partially folded conformation, stabilized by such specific contacts, also serves to create the necessary hydrophobic environment that, in addition to the capping motif, permits α-helix formation. In the final folded state of the protein, this substructure corresponds to the conserved GST motif II (14). The fact that capping mutations affect the thermal stability of mutants to a smaller extent with respect to their ability to refold suggests that the conformation of this intermediate during refolding is more labile than when present in the fully folded state. Considering that the GST motif II is a widespread conserved module also present in other protein structures (14), its involvement in folding and stability appears to be of a more general interest.

The S150A mutation does not affect the expression of the protein in E. coli at 37 °C. Thus, our in vitro folding system is much less efficient than protein folding in vivo. We may speculate about the presence in the host cell of a protein synthesis machinery, such as an ATP-dependent chaperone system, that facilitates the attainment of a more favorable equilibrium between folded and misfolded molecules. Once folded in the cell, the S150A mutant, but not the D153A and S150A/D153A mutants, is also stable at 37 °C as in vitro.

The helix capping hypothesis has already been proposed to explain how the primary structure influences the formation of an α-helix and thus affects protein stability and kinetics of folding (18–20, 28). The present results indicate that an N-capping box is essential for GST folding and stability. Its strict conservation in the hydrophobic core of the molecule as well as the results reported here represent novel observations for helix capping and permits more specific assumptions on the role played by this motif in protein folding. It has recently been hypothesized (29–30) that, for monomeric single-domain proteins, the two-state kinetics of folding follows a molecular growth mechanism. Buried and highly conserved residues, making a definite set of native-like contacts, determine the formation of a specific folding nucleus that represents a transition state of the folding process. The present results indicate that also in a more complex multidomain protein such specific nucleus interactions exist and that, in the GSTs, they are at least in part determined by a conserved capping box motif.

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