The Folding and Stability of Human Alpha Class Glutathione
Transferase A1-1 Depend on Distinct Roles of a Conserved
N-capping Box and Hydrophobic Staple Motif*

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An N-capping box and a hydrophobic staple motif are strictly conserved in the core of all known glutathione S-transferases (GST). In the present work, mutations of hGSTA1-1 enzyme residues forming these motifs have been generated. The analysis of S154A, D157A, and S154A/D157A capping mutants indicate that the removal of this local signal destabilizes the protein. The fact that the third helical residue D157A mutation (N-3) was much more destabilizing than the first helical residue S154A mutation (N-cap) suggests that the appropriate conformation of the conserved substructure formed by the α6-helix and preceding loop (GST motif II) is crucial for the overall protein stability. The refolding study of GSTA1-1 variants supports the prediction that this subdomain could represent a nucleation site of refolding. The analysis of L153A, I158A, L153G, and L153A/I158A hydrophobic staple mutants indicate that the removal of this motif destabilizes the GSTA1-1 structure as well as its refolding transition state. The hydrophobic staple interaction favors essential inter-domain contacts and, thereby, in contrast to capping interactions, accelerates the enzyme reactivation. Its strict conservation in the GST system supports the suggestion that this local signal could represent an evolutionarily conserved determinant for rapid folding.

It is a general observation that functionally and evolutionarily related proteins have a common overall architecture but divergent sequences. This means that not all features of the sequence are important for the attainment of the final conformation adopted by a protein. For monomeric single domain proteins it has been hypothesized (1, 2) and confirmed by direct experiments (3–5) that only a few non-functional residues are crucial to ensure fast and correct protein folding. In these cases the two-state kinetics of folding follow a molecular growth mechanism. Highly conserved residues of the hydrophobic core, making a definite set of native-like contacts, determine the formation of a specific folding nucleus onto which other parts of the molecule can grow. Mutations of residues forming these nucleus contacts are more deleterious for folding than others suggesting that the folding nucleus should be evolutionary conserved. This last phenomenon has been effectively observed in globins and cytochromes c families, and it has been proposed that likely it reflects a more general rule of protein folding (6, 7). Theoretical studies involving lattice calculations suggest that larger proteins can fold by multinucleation (8). Domains are folded separately, mimicking the folding of small proteins, and then dock to form the subunits, which in turn assemble to the native oligomeric structure (8). However, there are few experimental data, so far, to support the validity of the nucleation mechanism for the folding of large and complex proteins.

GSTs (9), of which many are homeric enzymes and thus represent the simplest oligomeric structure, have been found to be good model proteins for such specific folding studies. On the basis of several criteria the cytosolic GSTs have been grouped into different evolutionary classes (9, 10). Two domains form the subunit (11–16). The smaller N-terminal domain (domain I) adopts an α/β topology and provides most of the contacts with GSH. The larger C-terminal domain (domain II) is all-helical and contains some of the residues that form the hydrophobic binding site of the second substrate (14–17).

Alignment of all known GST structures (more than 100) shows that only 6–7 residues, i.e. less than 5% of the entire polypeptide chain, are strictly conserved. Despite this limited sequence similarity, all GSTs adopt the same native fold. GST structure analysis indicated that a limited number of residues, strictly conserved in the hydrophobic core, form two local structural motifs at the N-terminal region of the α6-helix (18–21). The N-capping box ((S/T)XXN-cap-N1-N2-N3-N4; where N1-N4 belong to the helix (Ser/Thr) and the N-3 (Glu/Asp) residues of an α-helix. (The nomenclature proposed by Richardson and Richardson (22) is: N-cap-N1-N2-N3-N4; where N1-N4 belong to the helix and N-cap is the boundary residue). The hydrophobic staple motif (22–27) is present in all soluble GSTs known today (18–21). The N-capping box (XXN-cap) and the hydrophobic staple motif (22–27) are present in all soluble GSTs known today (18–21, 27). The N-capping box (22, 27) is a recurring motif present at the beginning of helices, which has been proposed to influence the formation of the α-helix. This local signal consists of reciprocal main chain-side chain hydrogen bonds between the N-cap (Ser/Thr) and the N-3 (Glu/Asp) residues of an α-helix. (The nomenclature proposed by Richardson and Richardson (22) is: N’-N’-N-cap-N1-N2-N3-N4; where N1-N4 belong to the helix and N-cap is the boundary residue). The hydrophobic staple motif (25–27) involves a specific hydrophobic interaction between residues located at the N’ and N-4 positions. It has been proposed that when present in combination with a capping box (expanded box (27)), the hydrophobic staple motif gives a cooperative effect in defining the α-helix starting point (25, 26). However, in a statistical analysis (25) it has also been shown to

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1 The abbreviations used are: GST, glutathione S-transferase; GSH, glutathione; CDNB, 1-chloro-2,4-dinitrobenzene; GdnHCl, guanidine hydrochloride; DTT, dithiothreitol.
be present in the absence of a capping box, suggesting that it may represent an independent motif. Thus, the function of the hydrophobic staple motif in proteins and its possible independent role still remain to be clarified.

By using a peptide obtained by chemical synthesis, we found that in GST the conserved N-capping box has a crucial role in the helix formation of the isolated α6-peptide (18). The possible functions played by the N-capping box and the hydrophobic staple motifs in the intact protein have been studied in hGSTP1-1, a member of the Pi class GST (18–21). Studies on variants of hGSTP1-1 in which the capping residues have been substituted by alanine (19) indicated that the removal of this local motif greatly destabilizes the final structure of the protein as well as its folding pathway. However, the mutation of the capping residue at position N-3 (D153A) was more destabilizing than the N-cap mutation (S150A). Analysis of crystal structures of capping mutants, expressed at the permissive temperature of 25 °C (21), shows that the mutation of Asp153 results in greatly increased mobility of the loop preceding the α6-helix. This was especially due to the complete loss of an extensive hydrogen bonding network formed by the side chain of Asp153 with other loop residues that stabilize the conformation of this structural element. Thus, the local denaturation of this part of the protein destabilizes the entire molecule even at temperatures as low as 32 °C and generates temperature-sensitive folding mutants, unable to refold at subphysiological temperatures (19). The significance of the hydrophobic staple motif residues has been examined by site-directed mutagenesis in hGSTP1-1 (20). This hydrophobic interaction formed by a loop (N′) and helical residue (N-4), by restricting the conformation of the α6-helix relative to the α1-helix, favors the formation of essential interdomain contacts and accelerates the reactivation of the unfolded polypeptide chain. These motifs, together with Gly146, always located four residues before the catalytic histidine, may represent an independent motif. Thus, the function of the capping motif II (18–21, 28), formed by the α6-helix and the preceding loop. While the role of the conserved glycine still remains to be determined, mutations of residues forming the capping box and hydrophobic staple motifs are particularly deleterious for refolding of hGSTP1-1, indicating that this subdomain behaves as a specific folding nucleus whose formation is critical during the refolding of the dimeric GSTP1-1. However, in other GST forms additional local interactions may be present and the structural changes occurring upon mutation of these conserved motifs might be different. It is therefore not obvious that the roles attributed to these conserved motifs are valid in other members of the GST superfamily. Moreover, it is interesting to note that GST motif II, as an internalized module, is also conserved in the core of other proteins such as eukaryotic translation elongation factor (EF1γ), lignin-degrading β-etherase, and prionogenic yeast protein URE2 (28). Thus, studies aimed to investigate the universal role of these conserved residues have a broader interest.

In the present work the role played by the conserved capping box and hydrophobic staple motif in the folding and stability of hGSTA1-1, belonging to alpha class GSTs, has been investigated by a combination of protein engineering and structure/function analysis. The results show that also in a member of this GST class the conserved capping box and hydrophobic staple motifs maintain a critical role for protein folding and stability. In particular, the hydrophobic staple is an independent motif and represents an intrinsic and conserved determinant of the polypeptide chain for folding stability.

**EXPERIMENTAL PROCEDURES**

**Materials**—Wild-type human GSTA1-1 was obtained by expression of a cloned cDNA in *Escherichia coli* XL-1 Blue (Stratagene, La Jolla, CA) as previously described (29). GSH and 1-chloro-2,4-dinitrobenzene (CDNB) were purchased from Sigma.

**Construction of GSTA1-1 Mutants**—Site-directed mutagenesis was employed to generate capping box and hydrophobic staple mutants. The oligonucleotides used for the mutagenesis were: L153A, 5′-AACAGGGTACGGCCTGGTGACATCTGCTC-3′; L153G, 5′-AACAGGGTACGGCCTGGTGACATCTGGTC-3′; L153S, 5′-AACAGGGTACGGCCTGGTGACATCTGGTC-3′; L153G/I158A, 5′-AACAGGGTACGGCCTGGTGACATCTGGTC-3′; L153S/I158A, 5′-AACAGGGTACGGCCTGGTGACATCTGGTC-3′; L153G, 5′-AACAGGGTACGGCCTGGTGACATCTGGTC-3′. The oligonucleotides were phosphorylated before use in inverse polymerase chain reaction. The expression clone pKHAl (29) was used as a template. The polymerization reaction contained 0.8 μg of each of the mutagenic and the reverse primer, 0.2 mM dNTPs, 2.5 units of Pfu DNA polymerase (Stratagene, La Jolla, CA), and 50 μg of plasmid DNA that was used as a template. The polymerization reaction was initiated at 74 °C for 10 min and followed by 25 cycles of 94 °C for 1 min, annealing at 65–70 °C for 1 min, and 72 °C for 9 min. The polymerization reaction was terminated at 72 °C for 30 min. The reaction product was recovered from agarose gel after electrophoresis and the DNA was purified using the GeneClean kit (BIO 101, Inc., Vista, CA) and ligated in pKHAl (29) and used for transformation of *E. coli* XL-1 Blue cells. The entire cDNA encoding human GSTA1-1 was sequenced to verify that no undesired mutations had been introduced in the polymerase chain reaction.

**Protein Expression and Stability in the Host Cells as a Function of the Temperature of Growth Medium**— Cultures of *E. coli* XL-1 Blue containing plasmids were grown in 300 ml of LB broth in 2-liter Erlenmeyer flasks at 37 °C. At an *A*°<sub>600</sub> of about 0.35, 300 ml of isopropyl-1-thiogalactopyranoside was added to a final concentration of 0.2 mM. From the time of addition the culture was grown for about 20 h at 25 °C. The subsequent purification of wild-type and mutant proteins was performed as described previously (29) with the only modification that the enzymes were purified on a GSH-Sepharose affinity column (30). The intracellular protein yields of GST in bacteria were expressed as percentage of GST purified in relation to total cytosolic protein.

**Kinetic and Structural Studies on Wild-type and Hydrophobic Staple Motif Mutants Expressed at 25 °C**—The kinetic parameters, *k<sub>cat</sub>*, and *K<sub>m</sub>* were determined at 25 °C as previously described (31). The concentrations for GSH and CDNB were 2 and 1 mM, respectively. Spectroscopic properties of the mutants and the wild-type enzyme were also studied. A Jasco-600 spectrophotometer was used for CD measurements in the far-ultraviolet region from 200 to 250 nm. CD spectra were recorded at 0.3 mg/ml protein concentration with cuvettes of 0.1-cm path length in a thermostatted cell holder. Intracellular fluorescence spectra were measured with a Spex (model Fluoromax) spectrofluorometer. The excitation wavelength was 280 nm and the fluorescence intensity of emission spectra were analyzed at a protein concentration of 0.5 mg/ml.

**Heat Inactivation Assays**—Enzyme was incubated at each temperature for 10 min at a protein concentration of 0.1 mg/ml in 0.1 M potassium phosphate (pH 7.0) containing 1 mM EDTA and 5 mM DTT to prevent oxidative inactivation. The enzyme was heat inactivated in sealed Eppendorf tubes and the temperature was monitored with a Cryoskan telethermometer. The inactivation time courses were determined by following suitable aliquots at different time points from the denaturation mixture for assay of remaining activity. The activity was assayed in 0.1 M potassium phosphate (pH 6.5) with 2 mM GSH and 1 mM CDNB at 25 °C. In no case could the thermal inactivation be reversed by lowering the temperature of incubation.

**Temperature Dependence of Refolding in Vitro for Wild-type and Hydrophobic Staple Mutants**—When the refolding of human GSTA1-1 mutants was to be monitored, 10 μg enzyme was first denatured in 4 M guanidinium chloride (GdnHCl) (0.2 M phosphate, 1 mM EDTA, 5 mM DTT, pH 7.0) at 25, 32, 40, and 45 °C for 30 min and then diluted (defining time 0) 1:40 into renaturation buffer (0.2 M phosphate, 1 mM EDTA, 5 mM DTT, pH 6.5) at the same temperature. The final GdnHCl concentration was 0.1 M during refolding. All refolding experiments were carried out by rapid addition of the denatured enzyme to the renaturation buffer. Recovered activity was monitored as a function of
time by withdrawal of appropriate aliquots of the renaturation mixture followed immediately by dilution into 2.0 ml of assay buffer. Refolding rate constants were determined by non-linear regression analysis of the experimental data by using the KaleidaGraph 3.0.5 program (Abelbek, Software). Under our conditions, an equation describing a single exponential process can be fitted to the data, $A(t) = A(1 - \exp(-k_f t)) + B$, where $A(t)$ is the activity at time $t$, $A$ the amplitude, $k_f$ the rate constant, and $B$ the activity value at time 0. The effect of a mutation on the energy of the transition state of folding can be calculated using transition-state theory in a similar manner to that reported by Jackson et al. The stability of the transition state of a mutant protein relative to that of wild-type is calculated from $\Delta \Delta G_0 = RT \ln(k_f/k'_f)$, where $\Delta \Delta G_0$ is the difference in energy of the transition state of folding relative to the unfolded state between wild-type and mutant proteins; $k_f$ and $k'_f$ are the respective rate constants of folding.

The reactivation behavior of S154A, I158A, L153A, and L153G variants can be described by a single exponential equation. Thus, we have assumed: (i) that the enzyme reactivation follows, kinetically, a two-state model without intermediates so that the refolding rate, measured under native-like conditions, can be used to characterize the transition state for folding; (ii) that none of the mutations have any significant effect on the folding pathway, at least not when analyzed at subphysiological temperature. Refolded D157A, S154A/D157A, L153A/I158A, and L153G/I158A mutants were unstable even at 25 °C and not further considered in this study.

**Molecular Graphics Analysis—**Coordinates of GST x-ray structures were derived from the Brookhaven Protein Data Bank via the Anonymous file-transfer protocol. The crystal structures were analyzed by using Hyperchem (Autodesk, Sausalito, CA) and MolView 1.4.6 (Purdue University) programs. Figures were generated by using the RasMol (v2.6, 1994–1996, Roger Sayle) program.

**RESULTS**

**Identification of Conserved Local Motifs and Molecular Graphic Analysis—**At the N terminus of the α-helix located in domain II, the sequence (Ser/Thr)XX-Asp is strictly conserved in all GST classes. In particular, as shown in Fig. 1A, Asp$^{157}$ and Ser$^{154}$ in GSTA1-1 form a reciprocal side chain-main chain hydrogen bond network which is typical for a capping box motif. In addition to these interactions, Asp$^{157}$ forms a very favorable hydrogen bond with the backbone NH group of an hydrophobic residue (Leu$^{148}$) located in the long loop preceding the α-helix. A previous sequence alignment study (18) also revealed the presence of a secondary structural motif conserved at the N terminus of the α-helix of all known GSTs. This motif corresponds to the specific ($i, i+5$) hydrophobic interaction, named the hydrophobic staple motif, formed between residue N$, located before the first helical residue (Ser$^{154}$ in GSTA1-1, N-cap), and a residue N-4, located at position four within the helix. In GSTA1-1, the N$ and N-4 residues correspond to Leu$^{153}$ and Ile$^{158}$, respectively. It has been suggested that this motif, flanking the capping box, enhances the demarcation of the N terminus limit and serves to cause a reversal of the polypeptide chain direction as it forms the α-helix. Three-dimensional structure analysis of GSTs shows that the side chains of the hydrophobic residues at the N$ and N-4 positions are located within a distance of 4 Å from each other in all known GST structures and that the N$ and N-cap residues always adopt dihedral angles in the β-region of the Ramachandran plot. Thus, this secondary interaction possesses all structural characteristics of a hydrophobic staple motif and, despite the low overall sequence identity (in some cases less than 5%), is well conserved among GSTs and GST-related proteins. As shown in Fig. 1B, Ile$^{158}$, in addition to the hydrophobic staple interaction with Leu$^{151}$, may form very favorable hydrophobic contacts with Trp$^{21}$, Leu$^{22}$, and Ala$^{25}$ all belonging to an important structural element (α1-helix) of supporting the active site.

**Design, Expression, and Purification of Capping Box and Hydrophobic Staple Mutants of Human GSTA1-1—**To investigate the role of the conserved capping box motif, residues Ser$^{154}$ and Asp$^{157}$ in human GSTA1-1 were individually replaced with alanine producing the D157A, S154A, and S154A/D157A mutants by oligonucleotide-directed mutagenesis. To investigate the role of the hydrophobic staple motif in the folding process, site-directed mutagenesis was also used to produce single variants L153A and I158A as well as the double mutant L153A/I158A. However, considering that an alanine residue potentially can still form during the folding process, a weak hydrophobic staple and a glycine cannot, the L153G variant and the corresponding L153G/I158A double mutant were also generated. All variants of GSTA1-1 were expressed in E. coli and purified in a single chromatographic step using a GSH affinity matrix. No more than 10% of the total enzymatic activity remained unbound for any of the different GST variants. Apparently, the mutations do not affect the affinity for GSH-Sepharose. The purified proteins were homogeneous as judged by SDS-polyacrylamide gel electrophoresis (results not shown). Considering that some mutations could have resulted in temperature-sensitive species, protein expression of all mutants was performed at a growth temperature of 25 °C of the host cell. As shown in Table I, the yields of the D157A single variant and S154A/D157A, L153A/I158A, and L153G/I158A double mutants were very low (less than 1%). This, probably, reflects the fact that these variants fail to reach the native state even at a temperature below the physiological 37 °C.

**Kinetic and Structural Properties of Wild-type and Mutant**

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*Fig. 1. A, structural conformation of the α6-helix and the preceding loop in the crystal structure of the folded human GSTA1-1. Ser$^{154}$ and Asp$^{157}$ correspond to the N-cap and N-3 residues, respectively. Asp$^{157}$ forms an additional hydrogen bond, shown in the figure, with the backbone NH of Leu$^{148}$ located in the loop preceding the α6-helix. B, the hydrophobic staple motif in human GSTA1-1 is formed by the loop residue Leu$^{153}$ (N position) and the helical residue Ile$^{158}$ (N-4 position). Ile$^{158}$ can form hydrophobic interactions with Trp$^{21}$, Leu$^{22}$, and Ala$^{25}$. These last residues belong to the α1-helix which is an important structural element supporting the active site. The figures are based on the published crystal structure (16).*
Enzymes Expressed at 25 °C—Table I summarizes the kinetic parameters for the conjugation of CDNB with GSH catalyzed by wild-type GSTA1-1 and mutant variants. The \( k_{\text{cat}} \) and \( k_{\text{cat}}/K_m \) values for CDNB of S154A, I158A, L153A, and L153G variants, although to various extents, were all higher than those of the wild-type enzyme. In particular, mutants L153A and L153G displayed \( k_{\text{cat}} \) values about 2-fold higher as compared with the parent enzyme. The D157A variant displayed a \( K_m \) for GSH (0.075 mM) and a \( k_{\text{cat}} \) value (3 \( s^{-1} \)) significantly lower than those of wild-type. For all other mutants \( K_m \) determinations for GSH gave similar or slightly higher values than that of the wild-type. Kinetic parameters for S154A/D157A and L153G/I158A double mutants were not determined because these enzymes were unstable even at a temperature as low as 25 °C. The \( k_{\text{cat}}/K_m \) values for CDNB of the L153A/I158A mutant were 98 \( s^{-1} \) and 14.5 \( mM \), respectively (not shown). This estimated \( K_m \) value was about 7-fold higher than the highest concentration of CDNB possible to use in the assay mixture and was characterized by a very high standard error.

The S154A, I158A, L153A, and L153G mutants as well as the wild-type proteins, when expressed at 25 °C, displayed similar structural properties. The similar far-UV CD spectra of these variants as well as the same gel filtration retention times (not shown) indicate that the secondary structure and the dimeric arrangement of the proteins are essentially unaffected by these mutations. As shown in Fig. 1, Ile158, in addition to the hydrophobic staple motif (9), is critical for maintaining the native fold of GSTA1-1. Furthermore, the L153A/I158A double mutant was significantly more unstable than the wild-type and the corresponding single mutants, being almost completely inactivated after 10 min monitoring the residual activity after 10 min of exposure to different temperatures. As shown in Fig. 3, A and B, all mutants were destabilized compared with wild-type GSTA1-1. In particular, the S154A and D157A mutants were inactivated to more than 20 and 60%, respectively, at 50 °C, whereas the loss of activity of the wild-type enzyme under the same conditions, was less than 10% (Fig. 3A). These results suggest that single capping residue substitutions cause significant effects on the active site, even though it is situated far from the location of the mutations. The fact that mutant D157A was more unstable than mutant S154A also indicates that, in addition to the capping interactions, the buried hydrogen bond formed by Asp157 with the amide NH of Leu148 could also play an important role for the enzyme stability. At 50 °C the I158A and L153A single mutants (Fig. 3B) were about 90 and 10% inactivated. A simple explanation for this difference is provided by the analysis of the three-dimensional structure of hGSTA1-1. As shown in Fig. 1, Ile158, in addition to the hydrophobic staple interaction with Leu153 (13), interacts with amino acid residues Trp21, Leu22, and Ala23, belonging to an important structural element of the active site (the a1-helix). This suggests that these interdomain interactions are critical for the stability of GSTA1-1. Furthermore, the L153A/I158A double mutant was more unstable than the wild-type and the corresponding single mutants, being almost completely inactivated after 10 min exposure to physiological temperature. This suggests that the staple interaction has an important role for the stability of the folded protein. This result is in accordance with the observation that also the L153G mutant, in which the hydrophobic staple motif has been removed, was completely inactivated at 50 °C.

Reactivation Yields at Different Temperatures—The proposed role of the capping box and hydrophobic staple motifs in helical nucleation and in determining the nascent helix direction during folding, suggest that single substitution of residues that make up these local signals could affect protein refolding. To test this hypothesis, we have investigated the reactivation yields of GSTA1-1 and its mutants at different temperatures of reactivation. Each unfolded enzyme, denatured in 4 M GdnHCl for 30 min, was 40-fold diluted with phosphate buffer at pH 7.0 in the same denaturing tube to a final concentration of 0.1 M GdnHCl. The reactivation yields of capping mutants were different from that of the wild-type (Fig. 4). In particular, the
D157A variant was completely unable to refold at 25 °C even though the mutant enzyme produced at 25 °C was stable at the same temperature. On the contrary, at 25 °C, the wild-type and S154A mutant showed the same recovery of specific activity. However, by increasing the temperature of refolding toward physiological values also, the reactivation yield of the S154A variant was lower than that of the parent enzyme. It should be noted that most of the activity was recovered in a few minutes, in which time period the inactivation of this mutant is less than 5%. This suggests that the N-cap mutation not only slightly affects the stability of the folded protein, but, to higher extent, also destabilizes a productive intermediate of folding.

Fig. 3 shows the reactivation yields of wild-type GSTA1-1 and the hydrophobic staple mutants. The refolding yields of the L153A variant were very similar to those of the wild-type showing only small differences at physiological temperature. On the contrary, all other mutants, by increasing the temperature of refolding displayed much lower recoveries of activity as compared with the parent enzyme. In particular, already at 25 °C, the I158A variant was characterized by a lower refolding yield than that of wild-type and L153A mutant. Moreover the L153A/I158A double mutant, in which the hydrophobic staple motif has been largely removed, was completely unable to refold at 25 °C even though the folded enzyme was stable at this temperature.

The removal of the hydrophobic staple motif was also affected by the L153G mutation. The refolding yields of this variant was significantly dependent on the temperature. It was completely unable to refold at 40 °C and even at 32 °C it displayed a refolding yield significantly lower than that attained at 25 °C.

Analysis of Reactivation at 25 °C—A single exponential equation could be fitted to all data sets for the refolding kinetics. However, as shown in Table II, while the refolding rates of mutants S154A, I158A, and L153A were similar or only slightly different from that of the wild-type enzyme, the L153G variant, in which the staple motif has been significantly weak-
structure of GSTA1-1 indicates that Leu153, involved in the transition state of folding. However, the analysis of the folding process. The reactivation rates of all the other residues, as assayed for catalytic activity at 25 °C, were between 3 and 0.04 min⁻¹, and the corresponding ΔG‡ values were between 0.19 and 0.70 kcal/mol. These differences in refolding rates reflect changes in the free energy of activation of folding upon mutation, obtained from the corresponding k_ref values as described in the text.

**DISCUSSION**

An N-capping box and a hydrophobic staple motif are strictly conserved in the core of all known GSTs. In the present work, in order to verify the universal role played by residues forming these motifs, corresponding mutations to those previously done in hGSTP1-1 have been constructed for hGSTA1-1. This isoenzyme is a well characterized member of the alpha class GSTs showing 31.6% sequence identity with hGSTP1-1. GSTA1-1 is unstable at physiological temperature of 25 °C, on the overall stability of GSTP1-1 upon N-3 mutation (Table II). Since the N-cap makes several inter-domain contacts with α1-helix near the active site, these results suggest that single capping mutations that locally destabilize the conformation of the α6-helix, also affect in a direct fashion the stability of the active site. Moreover, the N-3 mutation (D157A) was much more destabilizing than the N-cap mutation (S154A). The GSTA1-1 structure provides a simple explanation for these results. As shown in Fig. 1A, the Asp157 residue, in addition to capping interactions, forms a very favorable hydrogen bond with the amide NH of Leu148. Thus, this internal hydrogen bond greatly increased the flexibility of the loop preceding the α6-helix. The higher mobility of the α6-helix and then for the overall protein stability. These results correspond well with those obtained for hGSTP1-1. In hGSTP1-1 the same internal hydrogen bond is formed between Asp153 and Ile144. We have found that the N-3 mutant (D153A) of the hGSTP1-1, expressed at the permissive temperature of 25 °C was unstable already at 35 °C. The crystal structure analysis of this variant indicated that the removal of the Asp153-Ile144 hydrogen bond greatly increased the flexibility of the loop preceding the α6-helix. The higher mobility of this structural element explains the dramatic effects, observed at 37 °C, on the overall stability of GSTP1-1 upon N-3 mutation. The fact that also the corresponding N-3 mutant of GSTA1-1 (D157A) is unstable at physiological temperature further supports the finding that in addition to capping box interactions, a general and very important role for the overall protein stability is played by this strictly conserved internal hydrogen bond. This interaction belongs to a network of loop-

| Wild-type | 0.51 ± 0.05 | ND |
| S154A | 0.37 ± 0.07 | ND |
| D157A | ND | ND |
| S154A/D157A | ND | ND |
| I158A | 0.48 ± 0.10 | -0.04 |
| L153A | 0.41 ± 0.03 | -0.17 |
| L153G | 0.17 ± 0.02 | -0.70 |
| I158A/L153A | ND | ND |
| I158A/L153G | ND | ND |

- ND, not detectable because the refolded enzymes were unstable at 25 °C.

![Graphs showing kinetics of reactivation at 25°C, 32°C, and 40°C.](http://www.jbc.org/)

Fig. 5. Kinetics of the reactivation of wild-type GSTA1-1 (●), I158A (△), L153A (●), and L153G (●), and L153A/I158A (○) hydrophobic staple mutants during the folding at different temperatures. Appropriate aliquots of enzyme, treated as described in the legend to Fig. 4, were assayed for catalytic activity at 25 °C.

TABLE II

| k_ref | ΔG‡ |
|-------|-----|
| min⁻¹ | kcal/mol |
| Wild-type | 0.51 ± 0.05 | ND |
| S154A | 0.37 ± 0.07 | ND |
| D157A | ND | ND |
| S154A/D157A | ND | ND |
| I158A | 0.48 ± 0.10 | -0.04 |
| L153A | 0.41 ± 0.03 | -0.17 |
| L153G | 0.17 ± 0.02 | -0.70 |
| I158A/L153A | ND | ND |
| I158A/L153G | ND | ND |

- ND, not detectable because the refolded enzymes were unstable at 25 °C.
helix bonds which are critical for stabilizing the loop conformation and it has been proposed that the loop and the subsequent α6-helix must be seen as a unique substructure previously referred to as GST motif II (28). It is interesting to note that this subdomain is also present, as a compartmentalized module, in other proteins such as eukaryotic translation elongation factor (EF1γ), dichloromethane dehalogenase, yeast nitrogen metabolism regulator (URE2), and lignin-degrading β-etherase. The results also show that the D157A variant is a temperature-sensitive folding mutant, unable to refold even at a temperature as low as 25 °C. An essentially similar finding was obtained with hGSTP1-1 indicating that the formation of the native-like loop-helix hydrogen bond is important during refolding. This also suggests that adopting the appropriate conformation of GST motif II is a critical step of the folding pathway and strongly supports the prediction that this subdomain could represent a nucleation site of folding.

To study the role of the conserved hydrophobic staple motif, L153A, I158A, L153G, and L153A/I158A variants of GSTA1-1, heterologously expressed at 25 °C of E. coli growth, have been generated. The results show that the presence of the staple interaction is important for the structural stability of GSTA1-1. This is in contrast with the observations made for a P1 class GST in which the removal of the hydrophobic staple interaction only has minor effects on the protein stability. Lacking additional structural information, it is only possible to conjecture that a different local environment or different local structural changes occurring upon mutation could compensate for the lack of this hydrophobic interaction in the P1 class GST.

The refolding yield of the I158A mutant at 25 °C was lower than that of the L153A mutant. Considering the interactions made by Ile158 with other residues of the α1-helix, these results suggest that the appropriate docking of the α6-helix with the α1-helix in domain I is also required for a productive folding pathway. In addition, the reactivation in vitro of the L153A/I158A double mutant was much lower than that found for either of the two L153A and I158A single mutation variants. In particular, the L153A/I158A double mutant was completely unable to refold at 25 °C, although the final structure of this mutant is stable at this temperature. This means that during refolding of GSTA1-1 the contribution from the staple motif is significant also in a situation where other interactions between Ile158 and the α1-helix occur. It should be noted also that the L153G variant shows a refolding yield lower than that of wild-type at 32 °C, while this mutant is stable at the same temperature. This means that the L153G variant, in which the hydrophobic staple motif has been removed, is a temperature-sensitive folding mutant and furthermore, confirms the critical role played by the staple interaction during refolding. In particular, the analysis of the reactivation rates at 25 °C indicates that the removal of the hydrophobic staple motif destabilizes the transition state of folding. The reactivation rate constant of the L153G variant, in contrast to other mutants, was about three times lower than that of the wild-type. This indicates that the staple interaction is formed at the transition state and decreases the activation energy for reactivation. It should be noted that the refolding rates of the capping mutants were similar to that of the wild-type. This means that the staple interaction plays an independent role during refolding, differently to the capping box motif, at longer times of reactivation. Considering the proposed role of this motif in defining a reversal of the polypeptide chain direction (25), the results suggest that the appropriate conformation of the α6-helix relative to the preceding loop, favors essential inter-domain contacts and thereby, accelerates the formation of the active site. Similar results have also been found in hGSTP1-1 suggesting that the hydrophobic staple motif could represent an evolutionarily conserved determinant of GST for rapid folding.

Here we show that the capping box and the hydrophobic staple motif, strictly maintained at the beginning of the α6-helix in the GST system, have important roles for folding which are conserved in two different members of GST. The information about the function of the staple interaction in proteins is limited. In particular, it is not known whether this local interaction can be considered as an independent motif. Here we provide evidence that, in GSTs, the capping box and hydrophobic staple motif play well defined but distinct roles during refolding.

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