Stabilization of Human Triosephosphate Isomerase by Improvement of the Stability of Individual α-Helices in Dimeric as well as Monomeric Forms of the Protein†

Véronique Mainfroid,‡ Shekhar C. Mande,§ Wim G. J. Hol,§,|| Joseph A. Martial,*,‡ and Karine Goraj‡

Laboratoire de Biologie Moléculaire et de Génie Génétique, Université de Liège, Institut de Chimie, B6, Sart Tilman B-4000, Belgium, Department of Biological Structure and the Biomolecular Structure Center, University of Washington, Box 357742, Seattle, Washington 98195-7742, and Howard Hugues Medical Institute, University of Washington, Box 357742, Seattle, Washington 98195-7742

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ABSTRACT: Human triosephosphate isomerase (hTIM) is a dimeric enzyme of identical subunits, adopting the α/β-barrel fold. In a previous work, a monomeric mutant of hTIM was engineered in which Met14 and Arg98, two interface residues, were changed to glutamine. Analysis of equilibrium denaturation of this monomeric mutant, named M14Q/R98Q, revealed that its conformational stability, 2.5 kcal/mol, is low as compared to the stability of dimeric hTIM (19.3 kcal/mol). The fact that this value is also lower than the conformational stabilities usually found for monomeric proteins suggests that the hTIM monomers are thermodynamically unstable. In the present work, we attempted to stabilize the M14Q/R98Q mutant by introducing stabilizing mutations in α-helices of the protein. Five mutations were proposed, designed to increase α-helix propensity by introducing alanines at solvent-exposed sites (Q179A, K193A), to introduce favorable interactions with helix dipoles (Q179D, S105D), or to reduce the conformational entropy of unfolding by introducing proline residues at the "N-cap" position of α-helices (A215P). Three replacements (Q179D, K193A, and A215P) were found to increase the stability of the native dimeric hTIM and the monomeric M14Q/R98Q. These results suggest that the monomeric hTIM mutant can be stabilized to a considerable extent by following well-established rules for protein stabilization. A comparison of the stabilizing effect performed by the mutations on the dimeric hTIM and the monomeric M14Q/R98Q allowed us to reinforce a model of equilibrium denaturation proposed for both proteins.

Considerable progress has been made over the last decade in understanding the important events that occur during the folding reaction of oligomeric proteins (Jaenicke, 1987, 1991; Janin, 1991; Jones & Thornton, 1995). A detailed description of this folding/assembly mechanism involves the characterization not only of the initial (denatured) and final (native) states of the oligomeric structure but also of all the intermediate states occurring during the quaternary structure formation. A great emphasis therefore has to be placed on the search for these intermediate states and on their analysis.

In most cases, the main intermediates occurring during the folding process of homodimeric proteins, which represent the simplest oligomeric structures, are the folded monomers. A useful technique for isolating these monomers from the native dimeric structure involves a disruption of the interactions between the monomers by site-directed mutagenesis experiments. This strategy has been successfully applied to the dimeric tyrosyl-tRNA synthetase (Jones et al., 1985), ribulose-1,5-bisphosphate carboxylase/oxygenase (Lee et al., 1987), λ-Cro (Mossing & Sauer, 1990), and glutathione reductase (Nordhoff et al., 1993).

In previous work, we engineered a monomeric mutant of human triosephosphate isomerase (hTIM),[1] a homodimeric enzyme, by introducing point mutations at the interface between the monomers (Mainfroid et al., 1996). TIM is the glycolytic enzyme which catalyzes the interconversion of D-glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (Rieder & Rose, 1959). It is a dimer of identical subunits, each of M_r 27 000. Both monomers contain a complete active site, but TIM is only active as a dimer, which suggests that residues from one monomer are important for maintaining the integrity of the active site of the other monomer (Wierenga et al., 1992). The TIM monomers adopt the α/β-barrel fold, in which eight β-strands form an internal parallel β-barrel surrounded by eight α-helices (Farber & Petsko, 1990; Brändén, 1991). Crystal structures of TIMs from chicken (Banner et al., 1975), yeast (Lolis et al., 1990), *Trypanosoma brucei* (Wierenga et al., 1991), *Escherichia coli* (Noble et al., 1993), human (Mande et al., 1994), and *Bacillus stearothermophilus* (Delboni et al., 1995) have been determined. An analysis of these structures reveals a fairly extensive interface between both monomers. One major area of contact between the two subunits is provided by an interdigitating loop, the interface loop (residues 65−79), that extends from one subunit and encircles the residue Met14 of the other subunit. Between these contacts is a polar pocket in which a number of salt bridges, hydrogen bonds, and solvent molecules play a significant role in the intersubunit interactions. Most of these interactions involve Arg98, a strictly conserved residue.

We have shown that simultaneously replacing the interface residues Met14 and Arg98 in hTIM with glutamine leads to

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* To whom correspondence should be addressed.

‡ Université de Liége.

§ Department of Biological Structure and the Biomolecular Structure Center, University of Washington.

|| Howard Hughes Medical Institute, University of Washington.

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[1] Abbreviations: TIM, triosephosphate isomerase; GuHCl: guanidium chloride; GAP, D-glyceraldehyde 3-phosphate.

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the dimer dissociation. An analysis of the equilibrium denaturation process of the monomeric hTIM mutant, named M14Q/R98Q, revealed that the protein is fairly unstable; its conformational stability, 2.5 kcal/mol, is lower than those usually found for monomeric proteins (Pace, 1990). This renders the protein manipulation and analyses rather critical and more or less rules out the crystallization attempts, which are favored by the use of sufficiently stable proteins. In the present work, we decided to apply site-directed mutagenesis to our hTIM monomer in an attempt to improve its stability.

Several strategies proposed for enhancing the stability of proteins (Fontana, 1991; Matthews, 1991; Fersht & Serrano, 1993) are based on introducing additional favorable interactions into the protein structure. Engineering electrostatic interactions (Hendsch & Tidor, 1994; Marqusee & Sauer, 1994; Waldburger et al., 1995), disulfide bridges (Eder & Wilmanns, 1992; Cho et al., 1994), improving α-helix propensity (Blaber et al., 1993, 1994), introducing favorable interactions with the α-helix dipole (Nicholson et al., 1988, 1991; Zhang et al., 1991; Chakrabarti, 1994), or exploiting conformational entropy differences between native and denatured states (Matthews et al., 1987; Herning et al., 1992; Nicholson et al., 1992; Ishikawa et al., 1993) have been used to increase the stability of proteins. More stable proteins could also be achieved by ion binding (Pantoliano et al., 1988; Kuroki et al., 1989), hydrophobic stabilization (Kellis et al., 1988; Matsumura et al., 1988; Sandberg & Terwilliger, 1991; Eriksson et al., 1992), eliminating internal cavities in the protein core (Kellis et al., 1988; Eriksson et al., 1992), or hydrogen bonding (Fersht et al., 1985; Shirley et al., 1992; Serrano et al., 1992a).

Another possible strategy for increasing the thermostability of proteins involves exchanging amino acids preferably occurring in mesophilic proteins with those preferably occurring in thermophilic proteins (Kelly & Brown, 1993). A comparison of the amino acid sequences and tertiary structures of mesophilic and thermophilic enzymes suggests that amino acid exchanges that increase hydrophobicity and decrease flexibility of α-helices are the main stabilizing factors of thermophilic enzymes (Menéndez-Arias & Argos, 1989). A common determinant cannot, however, be put forward as an explanation for the higher thermostability of all thermophilic enzymes, as each protein seems to possess its own stabilizing strategy.

A comparative study of the amino acid sequences of TIMs isolated from psychrotrophic, mesophilic, and thermophilic bacteria showed that an increase of the alanine content in α-helices, an increased number of favorable interactions with the α-helix dipoles, and a higher content of prolines and arginines could contribute to the fact that *B. stearothermophilus* TIM has a higher level of thermostability than its mesophilic and psychrotrophic counterparts (Rentier-Delrue et al., 1993). In an attempt to increase the stability of the monomeric hTIM mutant, we decided to focus on the three general stabilizing strategies that seem to be involved in thermoadaptation of TIMs, i.e., the introduction of favorable electrostatic interactions with α-helix dipoles, the increase of the alanine content in α-helices, and the increase of the proline content. Five different mutants were designed according to the wild-type hTIM structure (Mande et al., 1994). They arose from sequence comparisons with *B. stearothermophilus* TIM (Rentier-Delrue et al., 1993). The stabilizing effect of these five replacements, Q179D, S105D, Q179A, K193A, and A215P, as single or multiple mutants, was tested on wild-type hTIM and the M14Q/R98Q monomer. In this paper, we report the results obtained with both proteins and show that the monomeric and dimeric forms of hTIM may be stabilized to a great extent by appropriate point mutations.

MATERIALS AND METHODS

Site-Directed Mutagenesis: Production of the hTIM Mutants. Site-directed mutagenesis was performed on the hTIM cDNA cloned into bacteriophage M13mp19 using the U-DNA mutagenesis method of Kunkel (1985). The single hTIM mutants were obtained by using 21 bp oligonucleotides harboring the desired mutation, while the triple hTIM mutants were produced by simultaneously adding the three mutated oligonucleotides to the reaction mixture. The mutations were identified by sequencing each of the altered DNAs, and the cDNAs harboring the correct mutations were subcloned into the expression vector pARHS-3 (Mande et al., 1994). Expression was performed in *E. coli* BL21(DE3) cells using the T7 system (Studier & Moffatt, 1986). The dimeric hTIM mutants were produced at 37 °C as soluble proteins. The monomeric M14Q/R98Q/Q179D/K193A/A215P proved to be insoluble under these conditions and was produced in a soluble form by growing the bacterial culture at 28 °C. The mutant proteins were purified to homogeneity as previously described (Mande et al., 1994), and their purity was assessed by SDS−PAGE electrophoresis.

Production of B. stearothermophilus TIM. *B. stearothermophilus* TIM was produced and purified in the manner described by Rentier-Delrue et al. (1993).

Kinetic Studies. TIM activity was measured using a coupled-enzyme assay based on the decrease of absorbance at 340 nm due to oxidation of NADH. The assay was performed at 25 °C as described by Mainfroid et al. (1993).

Stability Studies. Fluorescence measurements were carried out at 25 °C with a Perkin-Elmer LS-50 spectrophotometer. Protein samples were excited at 290 nm, and the emission was recorded from 300 to 400 nm in 1-cm quartz cells. Bandwidths for excitation and emission were, respectively, 7 and 15 nm. Protein samples of 10 μg/mL in 50 mM Tris-HAc buffer, pH 8, were used. Denaturation of hTIM and its mutants was performed by urea, whereas guanidium chloride (GuHCl) was used for *B. stearothermophilus* TIM as urea was found to be inefficient in denaturing this protein. Protein samples were incubated for 24 h at 25 °C at each denaturant concentration prior to fluorescence measurements. This time period was sufficient to reach equilibrium as no more spectral changes were observed in the samples. Each spectrum was corrected by subtraction of the denaturant-containing buffer. The reversibility of denaturation was demonstrated by denaturing protein samples either in 8 M urea (hTIM mutants) or 6 M GuHCl (*B. stearothermophilus* TIM) for 24 h followed by dialysis. The full recovery of fluorescence characteristics and enzymatic activity was then checked for each protein sample. The thermodynamic parameters were calculated as previously described (Mainfroid et al., 1996), using the nonlinear least-squares program Enzfitter (Leatherbarrow, 1987). A two-state model of denaturation from a folded dimer to two unfolded monomers was used to analyze unfolding of dimeric TIMs, whereas the transition between a folded monomer and an unfolded

monomer was used to analyze unfolding of monomeric hTIM mutants.

RESULTS

Selection of Substitutions. The aim of this study was to improve the conformational stability of a hTIM monomeric mutant using site-directed mutagenesis. Because the monomeric M14Q/R98Q is not as easy to produce and to analyze as wild-type hTIM, we first decided to test the selected replacements on wild-type hTIM. We focused on amino acids which are not involved in intersubunit interactions. The amino acid substitutions were thus introduced in α-helices of the monomer which are not in contact with the other monomer. This is why α-helices 1, 2, and 3 were removed from consideration. No account was taken of sites where the side chain of a residue was found to participate in intramolecular interactions. The mutations were selected on the basis of the known hTIM crystal structure (Mande et al., 1994) and arose from sequence comparisons with *Bacillus stearothermophilus* TIM. Three stabilizing strategies were followed.

The first amino acid substitutions were designed to introduce favorable interactions with α-helix dipoles. We focused on the introduction of aspartic acid residues at the N-cap position of α-helices 4 and 6 of hTIM [according to the definition of Richardson and Richardson (1988)]. Charged side chains in the vicinity of these helices were closely observed, so that the suggested mutations do not create any imbalances in the local electrostatic environment of the helix dipoles. The selected substitutions, S105D (α4) and Q179D (α6), were modeled in the hTIM structure using Biograf (Vriend, 1990) and subsequently checked for possible collisions with neighboring residues. *B. stearothermophilus* TIM has a Glu at position 179, while yeast TIM has an Asp at position 105.

In a second attempt to increase hTIM stability, residues within α-helices were replaced with alanines. Table 1 lists all solvent-exposed residues in α-helices of hTIM. Located at the opposite site of the interface, α6 seems to be a good candidate for alanine replacements, hence our decision to select the Q179A and K193A mutations.

A third type of hTIM stabilization was investigated, in the light of amino acid substitutions that decrease the entropy of unfolding. We focused on the replacement of Ala215 (α7) with a proline as this residue, as well as the preceding glycine, have conformational angles that are within the allowed range for proline substitution (Schimmel & Flory, 1968). Ala215 has ϕ and ψ values of −78 and −49, respectively, whereas the preceding Gly214 has a ψ value of −35. Analysis of the wild-type hTIM structure suggests that Ala215 may potentially accommodate a proline without sterically interfering with neighboring atoms. *B. stearothermophilus* TIM has a proline at the N-cap position of α7. The positions of the five proposed replacements in the hTIM structure are shown in Figure 1.

Determination of the Kinetic Parameters of the Single hTIM Mutants. The five selected replacements, Q179D, S105D, Q179A, K193A, and A215P, were performed in hTIM as single mutations. The five mutants were tested for activity. Their kinetic parameters are listed in Table 2 and compared to those of the wild-type hTIM. The S105D replacement induced a 6-fold decrease in the catalytic efficiency of hTIM, whereas the four other replacements did not affect the enzyme activity.

Table 1: Solvent-Exposed Residues in α-Helices of hTIM Selected for Alanine Replacement[a]

residue	accessibility (%)[b]	α-helix	noncovalent interactions
Lys18	25	α1	Lys18−Asp349
Gln19	63	α1	Gln19−Glu23
Glu23	49	α1	Glu23−Gln19/Thr27
Thr27	33	α1	Thr27−Glu23
Asp49	6	α2	Asp49−Lys318
Gln53	49	α2	
Lys54	34	α2	
Glu107	61	α4	Glu107−Gln111
Gln111	30	α4	Gln111−Glu107
Glu140	38	α5	Glu140−Lys187
Lys141	51	α5	
Glu145	45	α5	
Lys148	39	α5	Lys148−Asp152
Asp152	50	α5	Asp152−Lys148
Gln179	53	α6	
Glu183	37	α6	Glu183−Lys187
Glu186	46	α6	
Lys187	37	α6	Lys187−Glu183/140
Lys193	44	α6	
Ser194	55	α6	
Asn195	46	α6	
Lys218	60	α7	
Ser222	41	α7	Ser222−Glu219
Asp242	46	α8	

[a] Sites already occupied by an Ala were not considered. [b] The solvent accessibility was calculated using the WHATIF program (Vriend, 1990).

Determination of the Conformational Stability of the Single hTIM Mutants. hTIM can be reversibly denatured by urea, and the denaturation process can be monitored by fluorescence spectroscopy (Mainfroid et al., 1996). The effect of each mutation on hTIM stability was therefore assessed by monitoring changes in the fluorescence emission peak wavelength as a function of urea concentration. All five mutants were found to unfold and refold reversibly. The unfolding curves are shown in Figure 2. In all cases, urea-induced denaturation resulted in a red-shifted emission peak wavelength from 323 to 344 nm, as was the case for wild-type hTIM. A 2-fold increase in the relative fluorescence intensity was generally observed, except for the S105D mutant, where the fluorescence intensity did not increase in a sigmoidal manner upon unfolding. A TIM concentration-dependent shift in the midpoint of the unfolding curves was observed for all five mutants, as already observed for wild-type hTIM. Data were analyzed according to a two-state model of denaturation based on the transition from a folded dimer to two unfolded monomers, as previously discussed in detail for hTIM (Mainfroid et al., 1996). This model can be described by

$$D_{NAT} \overset{K_D}{\leftrightarrow} 2M_{UNF} \qquad (1)$$

where D_{NAT} is the native dimer, M_{UNF} the denatured monomer, and K_D the equilibrium constant for the concerted dissociation/unfolding reaction.

The equilibrium constant and the free energy of denaturation are given by

$$K_D = [M_{UNF}]^2/[D_{NAT}] = 2P_t[f_d{}^2/(1 - f_d)] \qquad (2)$$

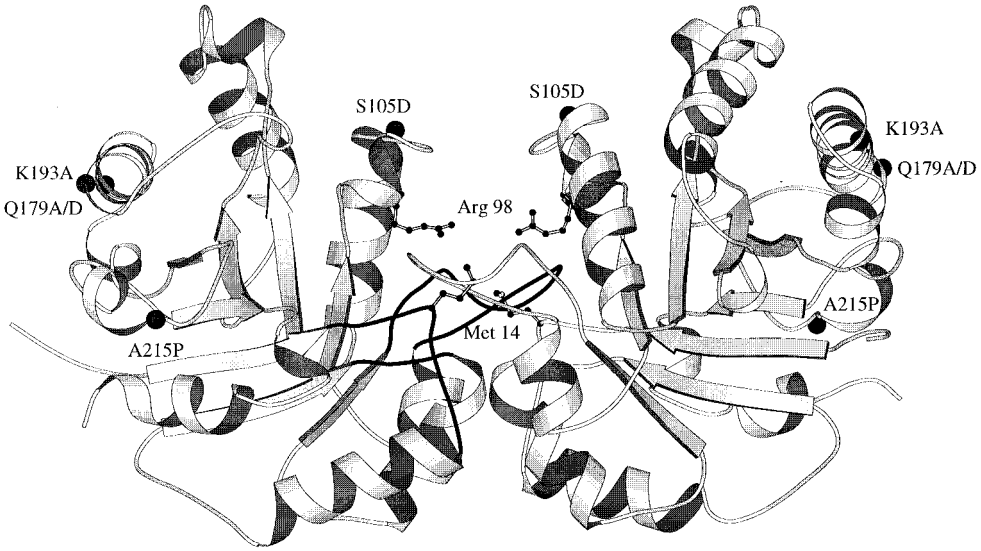

FIGURE 1: View of the Cα-backbone of the hTIM dimer. The α-helices are represented by spirals, and the β-strands by arrows. The side chains of Met14 and Arg98 from each monomer are shown at the interface of the dimer. The localization of the five replacements performed on hTIM, Q179D/A, S105D, K193A, and A215P, are visualized by black circles.

Table 2: Kinetic Parameters of hTIM Mutants[a]

	hTIM	Q179D	S105D	Q179A	K193A	A215P
K_m (GAP) (mM)	0.49	0.48	0.93	0.49	0.51	0.55
k_{cat} (GAP) (min^{-1})	2.7×10^5	2.7×10^5	8.2×10^4	2.69×10^5	2.67×10^5	2.49×10^5
k_{cat}/K_m (GAP) (min^{-1} mM^{-1})	5.5×10^5	5.62×10^5	8.8×10^4	5.49×10^5	5.24×10^5	4.53×10^5

[a] The values for wild-type hTIM are indicated for comparison. The assays were performed with 20 ng of protein.

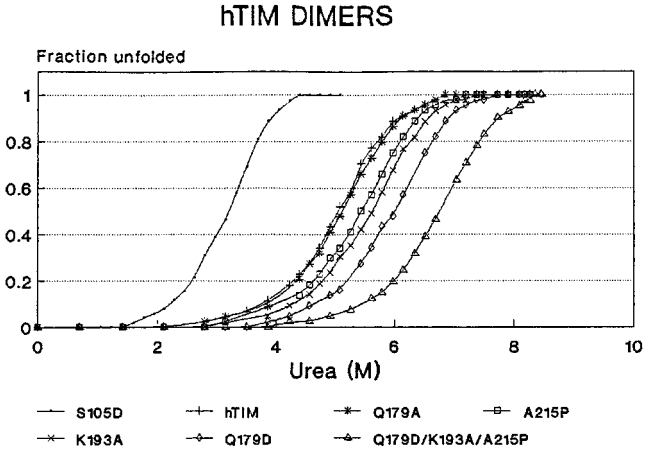

FIGURE 2: Urea-induced denaturation curves of wild-type hTIM and its single and triple mutants monitored by fluorescence spectroscopy. The fraction of unfolded protein at each urea concentration was calculated from the shift of the fluorescence emission peak wavelength, after correction for the pre- and posttransition baselines.

where P_t is the total protein concentration and f_d the fraction of unfolded protein.

$$\Delta G_D = -RT \ln K_D \quad (3)$$

The conformational stabilities of hTIM and its five mutants, calculated according to this model, are listed in Table 3. The Q179D, K193A, and A215P mutants were more stable than wild-type hTIM, with stabilities increased by 0.6−1.5 kcal/mol. The S105D replacement was clearly destabilizing, with a free energy of denaturation 1.3 kcal/mol lower than that of the wild type. The Q179A mutation did not affect the hTIM stability, as its conformational stability was essentially the same as wild-type hTIM.

Table 3: Thermodynamic Parameters of hTIM Mutants Determined by Urea-Induced Denaturation Monitored by Fluorescence

	ΔG_D (kcal/mol)[a]	m [kcal/(mol·M)][b]	$\Delta\Delta G_D$ (kcal/mol)[c]
hTIM	19.3 ± 0.4	1.72 ± 0.03	
Q179D	20.8 ± 0.4	1.68 ± 0.03	+1.5
S105D	18.0 ± 0.3	2.20 ± 0.05	−1.3
Q179A	19.4 ± 0.2	1.69 ± 0.02	+0.1
K193A	20.3 ± 0.4	1.68 ± 0.03	+1.0
A215P	19.9 ± 0.2	1.68 ± 0.02	+0.6
Q179D/K193A/A215P	22.3 ± 0.3	1.68 ± 0.02	+3.0

[a] ΔG_D is the free energy of unfolding at 25 °C in the absence of urea. [b] m is a measure of the dependence of ΔG_D on urea concentration. [c] $\Delta\Delta G_D$ is the change in free energy of unfolding of the considered mutant relative to wild-type hTIM. A positive value of $\Delta\Delta G_D$ indicates that the mutant is more stable than wild-type hTIM. The standard deviations are those given by the Enzfitter program (Leatherbarrow, 1987).

Determination of the Conformational Stability of B. stearothermophilus TIM (*B. st. TIM*). The hTIM mutations were selected through sequence comparisons with *B. st.* TIM, so the conformational stability of this protein was determined to provide a reference value. As urea was found inefficient for denaturing *B. st.* TIM, GuHCl was used as a denaturant. In these conditions, unfolding was reversible. GuHCl-induced denaturation resulted in decreased relative fluorescence intensity and a red-shifted emission peak wavelength from 321 nm in the native state to 348 nm in the denatured state. A TIM concentration-dependent shift in the midpoint of the denaturation curve was observed (data not shown), consistent with the two-state equilibrium described by eq 1. The conformational stability of *B. st.* TIM, calculated according to this two-state model, was 24.2 ± 0.4 kcal/mol.

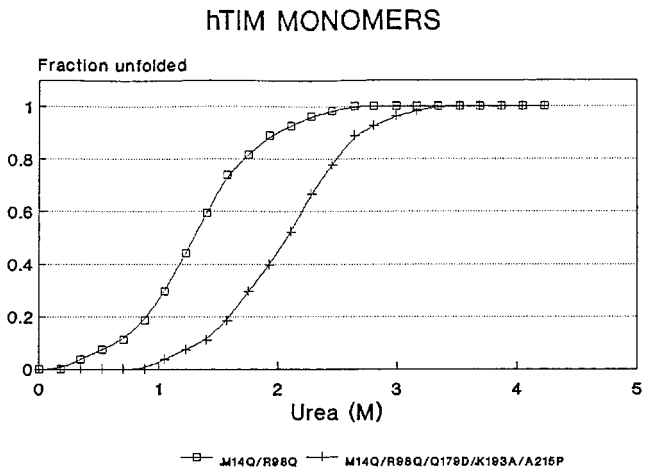

FIGURE 3: Urea-induced denaturation curves of monomeric hTIM mutants monitored by fluorescence spectroscopy.

There is a 4.9 kcal/mol increase in stability for the thermophilic *B. st.* TIM, as compared to the mesophilic hTIM.

Production of a Stabilized M14Q/R98Q hTIM Monomer. Three point mutations were found to significantly increase the stability of hTIM. The simultaneous effect of these three replacements, namely, Q179D, K193A, and A215P, was tested on the hTIM monomeric mutant, M14Q/R98Q, in which the replacement of two interface residues, M14Q and R98Q, prevents the association of the two monomers. The triple mutation was also performed on the hTIM dimer, so as to provide a comparative value.

The urea denaturation curve of hTIM harboring the triple mutation is shown in Figure 2. The conformational stability of this "stabilized hTIM dimer" was calculated according to the two-state model of denaturation (eqs 1−3) and was estimated at 22.3 kcal/mol (Table 3). The three stabilizing mutations thus increased the stability of hTIM by 3 kcal/mol. The urea-induced denaturation curve of the M14Q/R98Q mutant, in which the triple mutation was introduced, is shown in Figure 3. A protein concentration dependence of the unfolding equilibrium was not observed, since unfolding curves obtained with different protein concentrations were coincident (Figure 3). This is consistent with a model where only the folded monomer and the unfolded monomer are significantly populated at equilibrium, as already reported for the M14Q/R98Q hTIM monomer. The free energy of unfolding of the "stabilized hTIM monomer", harboring the three stabilizing mutations, was therefore analyzed according to a model of denaturation described by

$$M_{NAT} \overset{K_D}{\leftrightarrow} M_{UNF} \quad (4)$$

where M_{NAT} is the folded monomer and M_{UNF} the unfolded monomer.

The equilibrium constant and the free energy of unfolding are given by

$$K_D = f_d/(1 - f_d) \quad (5)$$

$$\Delta G_D = -RT \ln K_D \quad (6)$$

As shown in Table 4, the simultaneous introduction of the three stabilizing mutations Q179D, K193A, and A215P in the hTIM monomer increased its stability by 1.4 kcal/mol.

Table 4: Thermodynamic Parameters of Monomeric hTIM Mutants Determined by Urea-Induced Denaturation Monitored by Fluorescence Spectroscopy

	ΔG_D (kcal/mol)[a]	m [kcal/(mol·M)][b]	$\Delta\Delta G_D$ (kcal/mol)[c]
M14Q/R98Q	2.5 ± 0.1	1.97 ± 0.05	
M14Q/R98Q/Q179D/K193A/A215P	3.9 ± 0.1	1.89 ± 0.04	+1.4

[a] ΔG_D is the free energy of unfolding at 25°C in the absence of urea. [b] m is a measure of the dependence of ΔG_D on urea concentration. [c] $\Delta\Delta G_D$ is the change in free energy of unfolding of the considered mutant relative to wild-type hTIM. A positive value of $\Delta\Delta G_D$ indicates that the mutant is more stable than wild-type hTIM. The standard deviations are those given by the Enzfitter program (Leatherbarrow, 1987).

DISCUSSION

In the present study, protein engineering experiments were carried out on a monomeric mutant of hTIM, in an attempt to increase its conformational stability. Three types of stabilization were considered, based on α-helix dipole stabilization, increased α-helix propensity, and decreased entropy of unfolding of the polypeptide backbone. Five mutations were designed on the basis of the wild-type hTIM crystal structure, and three of them, namely, the Q179D, K193A, and A215P replacements, were found to be successful in increasing the stability of both dimeric and monomeric forms of hTIM. This work therefore provides new quantitative data on the numerous attempts designed to increase the stability of proteins by simple principles.

The most important stabilizing effect performed on the hTIM dimer was obtained with the Q179D replacement, which increased the protein stability by 1.5 kcal/mol. This corresponds well with the values reported for other proteins (Serrano & Fersht, 1989; Nicholson et al., 1991; Forood et al., 1993; Chakrabarti, 1994) and supports the general finding that amino acid substitutions designed to interact favorably with α-helix dipoles may represent a general strategy for enhancing the stability of a protein.

The A215P replacement was performed as an initial test for protein stabilization by decreased entropy of unfolding. This mutation proved successful, with a 0.6 kcal/mol increase in hTIM stability. This was the sole replacement proposed for entropic stabilization, as this type of substitution is highly dependent on the site of mutation itself and also on the structural context in which it occurs. The number of sites that meet all the criteria for proline replacements therefore seems limited, and only one preferred candidate was found in hTIM. Interestingly, the residue preceding Ala215 in hTIM is a glycine. Ueda et al. (1993) suggested that a glycine at the N-terminal position of a proline greatly reduces the possible strain caused by the introduction of a proline residue. Moreover, as a glycine in a polypeptide chain is entropically unfavorable, the best strategy for creating a Gly-Pro sequence is to utilize a glycine already present in the protein. By introducing a proline after the Gly214 in hTIM, we were therefore in a highly favorable situation.

The fact that the K193A mutation increased the stability of hTIM by 1 kcal/mol was attributed to the introduction within an α-helix of a good helix former, alanine. This value is similar to the changes in stability reported by other researchers (Daopin et al., 1990; Zhang et al., 1991). Unlike what is observed for proline substitutions, where relatively

few positions can be tested, many possible sites exist for alanine substitutions that may potentially be used for enhancing the stability of hTIM (Table 1). The alanine replacements proposed in this study may therefore be considered as first attempts of alanine stabilization, and other propositions may be formulated and assayed in a near future.

The results obtained with these three first mutants fit in with recent findings that genetically more stable proteins can be produced by improving the stability of individual α-helices. Nevertheless, two point mutations failed at increasing hTIM stability.

The Q179A replacement was found to be inefficient in stabilizing hTIM, as its conformational stability was fairly similar to that of wild-type hTIM, and some explanation for this may emerge from statistical analysis. The Q179A mutation was performed at the N-cap position of α6. However, Ala is rarely found at the caps of α-helices (Richardson & Richardson, 1988), and it was proposed that the side chains of alanine and larger residues at the N-cap do not satisfy hydrogen bonding requirements of the first four residues, and inhibit the solvation of the helix N-terminus (Serrano et al., 1992b,c). This could explain why the Q179A replacement failed at increasing hTIM stability. The Q179A mutation was not destabilizing, however, and this is probably because Gln itself is not favorable as N-cap residue, since it is unable to make hydrogen bonds with residues at positions N+2 and N+3, and is quite rare at the N-caps (Richardson & Richardson, 1988).

The S105D mutation, designed to create a favorable interaction with the N-terminus of α4 in hTIM, destabilized the protein by 1.3 kcal/mol. This was very unexpected, and we tried to determine if this destabilizing effect was due to the introduction at position 105 of an aspartic acid or to the removal of the initial serine. Oγ of Ser105 in hTIM makes a hydrogen bond with the main chain N of Leu108, and it was thought that replacing Ser105 with an Asp could remove this favorable interaction. This seems unlikely, however, as Asp residues are known to make similar hydrogen bonds in the first turn of helices (Richardson & Richardson, 1988). It is therefore probable that the destabilizing effect due to the S105D replacement was not produced by the substitution itself but by the structural context in which it occurred. The Asp residue was inserted at a site already containing many charges, as four charged amino acids are in close proximity to Ser105. In wild-type hTIM, two of them, Glu104 and Glu107, point in opposite directions, while the two others, Asp106 and Arg99, are involved in a salt bridge. None of these four charged residues is therefore supposed to produce unfavorable electrostatic interactions with the new Asp introduced at position 105. It should be borne in mind, however, that little is known about the possible structural rearrangements undergone by a mutation, and we cannot exclude the possibility that the substituted Asp105 generated unfavorable electrostatic interactions with some of its neighboring residues. The destabilizing effect due to the S105D substitution in hTIM could also be explained by the fact that the mutation has altered the polypeptide conformation and induced further structural changes. This may be relatively important as the residue preceding Ser105 is the highly conserved Glu104, which appears to be essential to the enzyme structure and stability (Daar et al., 1986). Learning about the three-dimensional structure of this mutant should help a great deal in understanding the cause of this unexpected destabilizing replacement.

In an attempt to stabilize the monomeric hTIM mutant, M14Q/R98Q, the three individual replacements found to increase the stability of the wild-type hTIM dimer, Q179D, K193A, and A215P, were simultaneously introduced into the monomeric mutant. The effect of the triple mutation was also tested on the hTIM dimer, so as to provide a comparative value.

Simultaneously performing the three individual stabilizing mutations, Q179D, K193A, and A215P, and analyzing their effect on the hTIM dimer revealed an almost additive effect, with a stability increased by 3 kcal/mol. This suggests that the three sites of substitution do not interact with one another. These results correspond well with the situation generally observed, when the stabilization arising from independent mutations is simply additive (Wells, 1990). The hTIM dimer harboring the three stabilizing mutations exhibits a conformational stability of 22.3 kcal/mol, which is quite close to the value obtained for the thermophilic *B. stearothermophilus* TIM, with a free energy of denaturation of 24.2 kcal/mol. This corresponds well with the general observation that thermophilic enzymes are stabilized in relation to their mesophilic homologues by the equivalent of a few noncovalent interactions (Fontana, 1988; Menéndez-Arias & Argos, 1989). We could therefore attempt to engineer a mutated hTIM dimer exhibiting a conformational stability comparable to that of the thermophilic *B. stearothermophilus* TIM, by introducing a few more stabilizing interactions in the Q179D/K193A/A215P hTIM mutant.

The triple mutation increased the stability of the hTIM monomer, M14Q/R98Q, by 1.4 kcal/mol, and the conformational stability of this "stabilized hTIM monomer" is therefore 3.9 kcal/mol. This stabilizing effect, although fairly limited, can be of relative importance for any further analysis of this mutant. Indeed, increasing the stability of the hTIM monomer allows easier production and analyses. Moreover, crystallographic assays are favored for proteins that display a significant conformational stability, since a lot of conditions has to be tested to find the good crystallization condition, and crystallization attempts are now underway with the monomeric M14Q/R98Q.

What is striking is the fact that the stabilizing effect produced by the same three replacements differs depending whether the mutations are performed on the wild-type hTIM dimer or on the M14Q/R98Q hTIM monomer. The simultaneous replacement of Q179D, K193A, and A215P increased the conformational stability of the hTIM dimer by 3 kcal/mol, while the stability of the M14Q/R98Q monomer was increased by 1.4 kcal/mol.

The apparent discrepancy between these values obtained on the hTIM dimer and monomer may be understood from an analysis of the equilibrium denaturation models proposed for both proteins. The model describing equilibrium denaturation of the hTIM dimer is two-state and can be described by eq 1. The conformational stability of the protein can be obtained from eq 3. In previous work (Mainfroid et al., 1996), we proposed that the free energy of unfolding of hTIM, ΔG_D, may be regarded as a simple combination of the free energy of dimer dissociation, ΔG_{DISS}, and the free energy of unfolding for the two monomers, ΔG_{UNF}, even if the folded monomers get masked in some experiments:

$$\Delta G_D = \Delta G_{DISS} + 2\Delta G_{UNF} \quad (7)$$

The sites where the stabilizing replacements were performed in the hTIM dimer were not involved in intersubunit interactions. Their effect on the conformational stability of hTIM is thus probably attributed solely to a change of ΔG_{UNF}, since ΔG_{DISS} is supposed to be unaffected. The stabilizing effect of the mutations, $\Delta\Delta G_{UNF}$, is correlated to the modification of conformational stability of the considered protein, $\Delta\Delta G_D$, by the following relationships:

$$\Delta G_D + \Delta\Delta G_D = \Delta G_{DISS} + 2\Delta G_{UNF} + 2\Delta\Delta G_{UNF} \quad (8)$$

$$\Delta\Delta G_D = 2\Delta\Delta G_{UNF} \quad (9)$$

Equilibrium denaturation of M14Q/R98Q was described by eq 4, and the conformational stability was calculated according to eq 6. For this monomeric protein, the stabilizing effect performed by the triple replacement, $\Delta\Delta G_{UNF}$, is correlated to the modification of conformational stability of the protein, $\Delta\Delta G_D$, by

$$\Delta G_D + \Delta\Delta G_D = \Delta G_{UNF} + \Delta\Delta G_{UNF} \quad (10)$$

$$\Delta\Delta G_D = \Delta\Delta G_{UNF} \quad (11)$$

According to our models (eqs 9 and 11), the effect performed by the triple replacement on the hTIM dimer is thus expected to be twice as big as that observed on the hTIM monomer. Our experimental values correspond well with this assumption. This suggests that our denaturation models are valid and that the conformational stability of the dimeric hTIM relative to its monomeric unfolded form, ΔG_D, may be obtained from a simple addition of its dissociation and unfolding components (eq 7).

It is likely that this relationship also holds true for other dimeric proteins whose denaturation is two-state, as already suggested for trp repressor (Fernando & Royer, 1992; Eftink et al., 1994) and β-nerve growth factor (Timm & Neet, 1992). This will greatly simplify thermodynamic calculations aimed at describing the denaturation process of such proteins.

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