Probing the Unfolding Region in a Thermolysin-like Protease by Site-Specific Immobilization†

Johanna Mansfeld,*,‡ Gert Vriend,§ Bertus Van den Burg,|| Vincent G. H. Eijsink,⊥ and Renate Ulbrich-Hofmann‡

Department of Biochemistry/Biotechnology, Martin-Luther University Halle-Wittenberg, Kurt-Mothes-Strasse 3, D-06120 Halle, Germany, EMBL, Biocomputing, Meyerhofstrasse 1, D-69117 Heidelberg, Germany, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands, and Department of Biotechnological Sciences, Agricultural University of Norway, P.O. Box 5051, 1432 Ås, Norway

Received January 4, 1999; Revised Manuscript Received March 30, 1999

ABSTRACT: Protein stabilization by immobilization has been proposed to be most effective if the protein is attached to the carrier at that region where unfolding is initiated. To probe this hypothesis, we have studied the effects of site-specific immobilization on the thermal stability of mutants of the thermolysin-like protease from *Bacillus stearothermophilus* (TLP-ste). This enzyme was chosen because previous studies had revealed which parts of the molecule are likely to be involved in the early steps of thermal unfolding. Cysteine residues were introduced by site-directed mutagenesis into various positions of a cysteine-free variant of TLP-ste. The mutant enzymes were immobilized in a site-specific manner onto Activated Thiol-Sepharose. Two mutants (T56C, S65C) having their cysteine in the proposed unfolding region of TLP-ste showed a 9- and 12-fold increase in half-lives at 75 °C due to immobilization. The stabilization by immobilization was even larger (33-fold) for the T56C/S65C double mutant enzyme. In contrast, mutants containing cysteines in other parts of the TLP-ste molecule (N181C, S218C, T299C) showed only small increases in half-lives due to immobilization (maximum 2.5-fold). Thus, the stabilization obtained by immobilization was strongly dependent on the site of attachment. It was largest when TLP-ste was fixed to the carrier through its postulated unfolding region. The concept of the unfolding region may be of general use for the design of strategies to stabilize proteins.

The immobilization of proteins on surfaces is one of the most effective approaches for their stabilization. The mechanism of stabilization, however, is still poorly understood, and, usually, the success of any method is more a matter of trial and error than the result of rational concepts. Studies on the thermal inactivation kinetics of immobilized enzymes (*1*−*3*) and on the thermal unfolding of appropriately labeled (*3*−*5*) immobilized enzymes, by fluorescence and ESR, have led to the conclusion that the stabilization effect due to immobilization strongly depends on the position of the attachment. It has been hypothesized (*2, 3*) that the stabilization by immobilization is large if that region of the molecule is fixed where under denaturing conditions unfolding is initiated, whereas it may be insignificant if the molecule is coupled to the carrier at sites remote from this "unfolding region". To prove this hypothesis, we have chosen the thermolysin-like protease (TLP)[1] from *Bacillus stearothermophilus* (TLP-ste) because in this enzyme the importance of local structural regions for thermal stability has been well recognized (*6, 7*) and knowledge about the initial phase of thermal unfolding is available.

Like for other broad-specificity proteases (*8*), thermal inactivation of TLP-ste is an irreversible first-order process whose rate is determined exclusively by the rate of partial unfolding processes that render the protein susceptible to autolysis (*6, 9, 10, 11*). For TLP-ste it has been shown that the surface-located region between residues 56 and 69 is crucial for thermal stability *(6, 7, 12)*, and that this region plays a predominant role in the partial unfolding processes that bring TLP-ste into an autolytically susceptible state *(6, 7, 12, 13)*. Thus, the 56−69 region must be one of the earliest to unfold upon heating and might be considered as "unfolding region" in the sense of the model described above.

The importance of local unfolding processes in a number of biologically significant processes has recently been recognized and attracted increasing attention. Unfolding regions have been localized in the SH3 domain of hematopoietic cell kinase (*14*), tropomyosin (*15*), ribonuclease A (*16*), and ribonuclease B (*17*). It has also been shown that aggregation leading to irreversible denaturation/inactivation of proteins is likely to be triggered by partial, as opposed to global, unfolding processes (*18*). In addition, local unfolding processes are exploited in the method of limited proteolysis, being an invaluable tool in studying protein structure and function (*19*).

† This work was supported by a research grant of the Leopoldina, the Deutsche Akademie der Naturforscher, and by the Deutsche Forschungsgemeinschaft, Bonn.

* To whom correspondence should be addressed. E-Mail: Mansfeld@biochemtech.uni-halle.de.

‡ Martin-Luther University Halle-Wittenberg.

§ EMBL Biocomputing.

|| University of Groningen.

⊥ Agricultural University of Norway.

[1] Abbreviations: DTT, 1,4-dithio-DL-threitol; TLP, thermolysin-like protease; TLP-ste, thermolysin-like protease from *Bacillus stearothermophilus*.

10.1021/bi990008p CCC: $18.00
Published on Web 06/10/1999

Probing the importance of the unfolding region in TLP-ste for stabilization by immobilization requires a uniform, controlled orientation of the enzyme on the surface. Such site-specific immobilization is usually achieved by covalent binding of the protein via single, mostly genetically engineered cysteine residues (*20*−*25*) or the reducing ends of carbohydrates in case of glycoproteins (*26*). Furthermore, noncovalent binding via His-tags (*27*), Arg-tags (*28*), biotin/streptavidin interactions (*29, 30*), or metal-chelating Langmuir−Blodgett films (*31*) can be used for this purpose.

In the present study, cysteine residues were introduced by site-directed mutagenesis at different positions in a cysteine-free TLP-ste variant (C288L) (*32*). The mutant enzymes were covalently immobilized via the introduced thiol groups onto polymeric carriers. The stabilization obtained upon immobilization via residues in the proposed unfolding region was compared with the stabilization obtained upon immobilization via residues in other parts of the molecule.

EXPERIMENTAL PROCEDURES

Reagents. 1,4-Dithio-DL-threitol was purchased from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany). 5,5′-Dithiobis(2-nitrobenzoate) was from SERVA Feinbiochemica GmbH (Heidelberg, Germany). Activated Thiol-Sepharose 4B and Thiopropyl-Sepharose 6B were purchased from Amersham Pharmacia Biotech Europe GmbH (Freiburg, Germany). Thermolysin was from Calbiochem-Novabiochem GmbH (Bad Soden, Germany). Casein was from MERCK (Darmstadt, Germany).

Plasmids and Strains. Plasmid pGE530 (*32*) contains the gene encoding the C288L variant of TLP-ste. The protease-deficient strain *Bacillus subtilis* DB117 (*33*) was used as host for this plasmid and variants thereof. Cells harboring these plasmids were grown at 37 °C in TY broth, containing 5 μg/mL chloramphenicol as antibiotic. In the case of the T56C/S65C mutant enzyme, 20 mM β-mercaptoethanol was added to the growth medium.

The *E. coli* strains WK6mutS and WK6 (*34*) as well as XL-1 Blue MRF′ (Stratagene GmbH, Heidelberg, Germany), and the plasmids pMa (*35*), PCR-Script Amp SK(+), and pBluescript II SK(+) (Stratagene) were used in site-directed mutagenesis procedures.

Design of Mutant Enzymes. Mutant enzymes derived from the cysteine-free variant of TLP-ste C288L (called pseudo-wildtype throughout this study) whose stability is nearly identical to that of TLP-ste were designed using a three-dimensional model of TLP-ste described previously (*6*). Model building and structure prediction were done using the program WHAT IF (*36*). Residues are numbered throughout this paper according to the sequence of thermolysin (*37*).

Site-Directed Mutagenesis. The plasmid pGE530 was used as template for the construction of the S65C, N181C, T299, and S218C single mutants by site-directed mutagenesis via PCR (megaprimer method) (*38*). PCR fragments were cloned into pBluescript II SK(+) or PCR-Script Amp SK(+) (Stratagene), and restriction mapping was used to screen for mutant clones (the mutagenic primers were designed such as to delete or introduce a restriction site). The T56C mutant was constructed using the pMa/c system (*34, 35*) as described previously (*32*). The sequence of mutated DNA fragments was verified by dideoxy sequencing (*39*) before using the fragments to construct variants of pGE530 containing mutated forms of the TLP-ste gene. The construction of the G8C and N60C mutants has been described earlier (*12*). The double mutant N60C/N181C was obtained by cutting and ligating appropriate fragments of the corresponding single mutant genes N60C and N181C. The double mutant T56C/S65C was obtained by PCR as described above, using the T56C variant of pGE530 as template.

Expression and Purification of Pseudo-Wildtype and Mutant Enzymes. Pseudo-wildtype and mutant proteins were produced and purified as described previously (*33, 40*) using affinity chromatography on Bacitracin-silica. The purification of the Cys-containing mutant enzymes was performed in the presence of 20 mM β-mercaptoethanol. The electrophoretically homogeneous enzymes were stored in elution buffer containing 20 mM sodium acetate, pH 5.3, 5 mM $CaCl_2$, 2.5 M NaCl, 20% (v/v) 2-propanol, 0.03% (w/v) sodium azide, and, only in case of the Cys-containing mutant enzymes, 20 mM β-mercaptoethanol. Immediately prior to use for immobilization, salts, β-mercaptoethanol, and 2-propanol were removed from the mutant enzymes by gel permeation chromatography using a Sephadex G25 superfine column (Amersham Pharmacia Biotech) and 20 mM sodium acetate, pH 5.3, 5 mM $CaCl_2$.

Prior to stability measurements of soluble enzymes, the purified TLP-ste variants were desalted by gel permeation chromatography on a Superdex75 HiLoad column (Amersham Pharmacia Biotech) in 0.05 M Tris buffer, pH 7.5, 5 mM $CaCl_2$.

Immobilization on Activated Thiol-Sepharose 4B and Thiopropyl-Sepharose 6B. Three hundred milligrams of Activated Thiol-Sepharose 4B or Thiopropyl-Sepharose 6B, respectively, was swollen according to the manufacturer's instructions and washed 3 times with the coupling buffer (20 mM sodium acetate buffer, pH 5.3, 5 mM $CaCl_2$). Twelve milliliters of the desalted enzyme solutions containing 0.2−0.8 mg of protein in 20 mM sodium acetate, pH 5.3, 5 mM $CaCl_2$ was incubated with the swollen gel. The suspension was stirred at 4 °C for 6 h. The gel was washed 3 times with 10 mL of 0.05 M Tris buffer, pH 7.5, 5 mM $CaCl_2$, 5 times with 10 mL of 0.05 M Tris buffer, pH 7.5, 5 mM $CaCl_2$, 1 M NaCl, and several times with 10 mL of 0.05 M Tris buffer, pH 7.5, 5 mM $CaCl_2$ until no protein was detectable in the filtrates. The enzyme−carrier complex was stored at 4 °C in 0.05 M Tris buffer, pH 7.5, 5 mM $CaCl_2$.

Enzyme Assays. Enzyme activities of the soluble enzymes were determined with an assay using 0.5% casein in 0.05 M Tris buffer, pH 7.5, 5 mM $CaCl_2$ at 37 °C for 30 min as described previously (*41*). The assay was calibrated using a standard wild-type enzyme preparation, and an arbitrary unit for protease activity was defined as the amount of activity required to increase the absorbance at 275 nm by 1 per minute under the conditions of the assay (*42*).

Activities of the immobilized enzymes were determined in a water-jacketed vessel containing a glass filter and a valve below the bottom filter; 30−50 mg of wet gel (1−3 mg dry weight) was incubated under stirring with 3 mL of a 0.5% (w/v) casein solution in 0.05 M Tris buffer, pH 7.5, 5 mM $CaCl_2$ for 30 min at 37 °C. The reaction was stopped by sucking off the solution from the immobilized enzyme. This solution was diluted appropriately, and further treated as described above for the soluble enzymes (*41*).

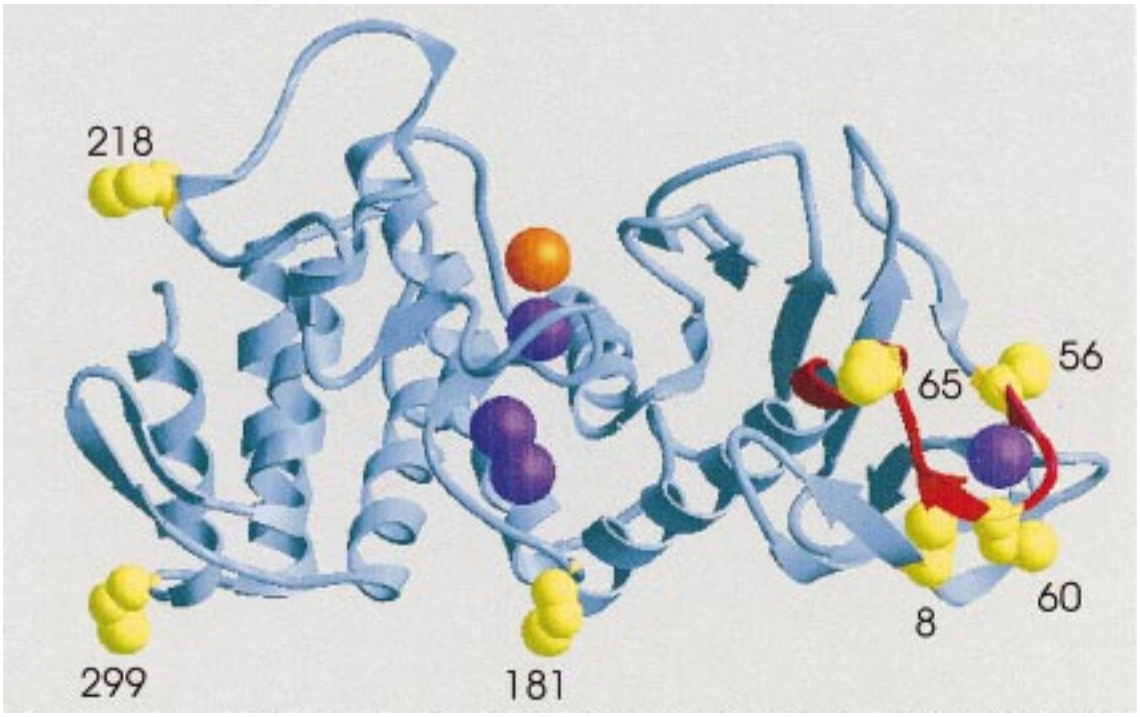

FIGURE 1: Positions of cysteine residues introduced into TLP-ste. The backbone is shown in blue. The unfolding region between residues 56 and 69 is drawn in red. The large spheres indicate the Zn^{2+} ion (orange) in the active site and the four Ca^{2+} ions (purple) bound to the molecule. The positions of the various cysteine residues introduced are shown in yellow. In this study, each mutant contained only one or two Cys.

Thermal Inactivation. The time course of thermal inactivation of the soluble enzymes was followed at pH 7.5 (0.05 M Tris buffer, pH 7.5, 5 mM $CaCl_2$) in the absence and presence of 10 mM DTT at 75 °C. Aliquots removed after different time intervals and cooled on ice were assayed for residual activity as described above. The dependence of thermal inactivation on enzyme concentration was checked for the G8C and T56C single mutant enzymes in the range of 0.3−4.0 μg of protein/mL.

For the determination of the thermal inactivation of the immobilized enzymes, samples of 30−50 mg of wet gel were incubated with 2 mL of 0.05 M Tris buffer, pH 7.5, 5 mM $CaCl_2$ at 75 °C for defined time intervals, cooled on ice, and assayed for residual activities as described above. The dependence of thermal inactivation on the amount of bound protein was examined for the immobilized mutant enzymes S65C in the range of 0.3−0.9 mg of protein/g of dry carrier and N181C in the range of 0.6−1.1 mg of protein/g of dry carrier.

The half-lives given are the averages of values resulting from at least two series of independently prepared batches of immobilized enzymes with at least 7 data points per series. The errors in the half-live values are less than 4% of the value.

Protein Determination. Protein concentrations were determined with the Bradford assay using bovine serum albumin as standard (*43*). The protein content of the immobilized enzyme and the coupling yields were calculated from the difference of the protein contents in the enzyme solutions before and after coupling.

RESULTS

Design and Production of the Mutants. To select suitable positions for the introduction of cysteine residues, a three-dimensional model of TLP-ste *(6, 36)* was used. This model was constructed on the basis of the crystal structure of the highly similar thermolysin (86% sequence identity) (*44, 45*). The model was previously shown to be sufficiently accurate for the successful design of various types of stabilizing mutations (*6, 12, 46, 47*). Accessibility of the introduced cysteines to functional groups on the carrier as well as accessibility of the active site cleft after immobilization were prerequisites. Mutations expected to result in significant clashes, unfavorable interactions, or other negative side effects were excluded. To minimize the risk of modeling errors, the Xxx→Cys mutations were only chosen in regions where TLP-ste and thermolysin are highly similar. As result, the single mutations T56C, S65C, N181C, S218C, and T299C (Figure 1) were selected. Furthermore, the already available single mutants G8C and N60C, which were designed in a previous study aimed at the introduction of a disulfide bridge in TLP-ste (*12*), were included in this study. In addition, the double mutants T56C/S65C and N60C/N181C were constructed.

All TLP-ste variants could be expressed in *B. subtilis* DB117. Expression levels were comparable to those of the wild-type enzyme, with the exception of the single mutant N60C and the double mutant N60C/N181C, for which the levels were about 3 times lower. The double mutant enzyme T56C/S65C could be produced in an active form only if β-mercaptoethanol was added to the growth medium. Purified pseudo-wildtype and mutant enzymes had similar specific activities (Table 1) toward casein as substrate at 37 °C, pH 7.5, showing that the selected mutations had no drastic effects on the catalytic properties of the soluble enzyme. Aggregation of the protein could be excluded under the conditions used as examined by gel filtration and analytical ultracentrifugation (results not shown).

Table 1: Immobilization of TLP-ste Variants on Activated Thiol-Sepharose and Thiopropyl-Sepharose

mutant	spec. act. of soluble enzyme [units/mg of protein]	amount of bound protein (mg/g of dry carrier)	coupling yield (%)	spec. act. of immobilized enzyme [units/mg of protein]
		Activated Thiol-Sepharose		
G8C	73.9	0.32	22.5	67.5
T56C	68.0	1.50	75.0	50.8
N60C	69.2	0.33	41.5	52.0
S65C	78.2	0.90	56.8	56.9
N181C	67.0	1.03	43.1	50.2
S218C	65.6	1.20	64.0	33.7
T299C	62.6	0.23	28.7	35.7
N60C/N181C	68.9	0.45	31.1	46.4
T56C/S65C	65.2	1.48	75.0	46.6
pseudo-wildtype	79.5	0.03	0.3	nd
thermolysin	nd	0.09	0.6	nd
		Thiopropyl-Sepharose		
pseudo-wildtype	79.5	0.6	67.7	nd
thermolysin	nd	0.2	21.9	nd

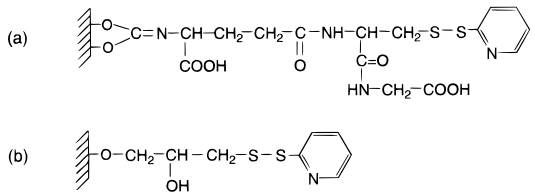

FIGURE 2: Functional groups of Activated Thiol-Sepharose 4B (a) and Thiopropyl-Sepharose 6B (b) for protein binding via SH groups.

Site-Specific Immobilization of the Mutant Enzymes. As carrier materials for site-specific immobilization of the various TLP-ste variants, Activated Thiol-Sepharose and Thiopropyl-Sepharose (Figure 2) were tested. The results of immobilization are shown in Table 1. Since Thiopropyl-Sepharose was found to bind also the pseudo-wildtype enzyme and thermolysin, both not containing any Cys residue, this carrier was excluded from further studies. Activated Thiol-Sepharose permitted the reversible, site-specific immobilization of the enzymes via their engineered thiol groups. With this carrier material, unspecific binding effects could be reduced to a minimum as indicated by the marginal coupling yields of pseudo-wildtype TLP-ste and thermolysin (Table 1). Coupling yields for the mutant enzymes were between 22 and 75 %, and the amount of bound protein was between 0.2 and 1.5 mg of protein/g of dry carrier material (Table 1). The specific activities of the mutant enzymes were between 51 and 91% of those of the soluble enzymes (Table 1). The activity was linearly dependent on enzyme amount bound to the carrier as examined for immobilized S65C and N181C in the range of 0.3−0.9 mg of protein/g of dry carrier and 0.6−1.1 mg of protein/g of dry carrier.

Thermal Stability. The thermal inactivation of all TLP-ste variants followed first-order kinetics, regardless of whether they were soluble or immobilized onto Activated Thiol-Sepharose. Thermal inactivation was shown to be independent of enzyme concentration or the amount of bound enzyme. The stability of the soluble mutant enzymes containing one or two surface-located cysteines may be affected by the formation of intermolecular disulfide bridges (*12*) or other types of cysteine oxidation (*48*). Therefore, stabilities of the soluble enzymes were determined in the absence and presence of 10 mM DTT. The results (Table 2) show that the thermal stabilities of the soluble enzymes were influenced to some extent by the Xxx→Cys mutation. Addition of DTT increased the thermal stabilities of most mutant enzymes considerably, indicating that oxidation of the cysteines indeed affected thermal stability. The differences in thermal stabilities of the soluble enzymes became smaller under reducing conditions.

Table 2: Thermal Stabilities of TLP-ste Variants Soluble and Immobilized to Activated Thiol-Sepharose

mutant	half-life of soluble enzyme (min)		half-life of immobilized enzyme (min)	ratio of half-lives of immobilized and soluble enzymes[a]
	in absence of DTT	in presence of DTT		
G8C	2.1	5.5	2.8	0.5
T56C	10.3	25.9	244.4	9.4
N60C	0.9	4.3	4.6	1.1
S65C	7.0	5.6	66.6	11.9
N181C	7.9	15.8	41.2	2.6
S218C	8.1	11.6	21.5	1.8
T299C	3.0	6.4	11.5	1.8
T56C/S65C	7.9	8.2	267.5	32.6
N60C/N181C	1.6	3.6	8.9	2.5
pseudo wild-type	7.1	7.3		

[a] Half-lives of the soluble enzymes in the presence of DTT were used for calculation.

The immobilization of the mutant enzymes affected thermal stability to very different extents (Table 2). Evident stabilization due to immobilization was found for the mutant enzymes T56C and S65C where the half-lives compared to the half-lives of the soluble enzymes (under reducing conditions) were increased 9 and 12 times, respectively. Even higher (33-fold) was the stabilization obtained upon immobilizing the T56C/S65C double mutant enzyme (Table 2). In contrast, immobilization of the mutant enzymes N181C, S218C, and T299C yielded only 1.8−2.6-fold stabilization. For the two mutant enzymes that were not primarily designed for immobilization purposes, carrier binding resulted in unchanged (N60C) or even reduced (G8C) stability (Table 2). Even combining N181C and N60C did not result in any further increase in the stabilization obtained upon immobilization.

DISCUSSION

The engineered TLP-ste variants containing Xxx→Cys mutations at different surface positions of the protein

molecule, which were site-specifically immobilized onto Activated Thiol-Sepharose via their thiol groups, proved to be appropriate to probe the influence of the attachment site in carrier binding despite the fact that the experimental approach suffers from some limitations. Thus, the stability of the Cys-containing soluble mutant enzymes is measured in the presence of DTT because some of the enzymes showed a strong tendency to oxidation, whereas the immobilized derivatives have to be measured without DTT due to the reversibility of enzyme−carrier binding. The protein contents of the immobilized mutant enzymes cannot be adjusted to exactly the same values because of different accessibilities and, possibly, reactivities of the differently localized Cys residues in the mutant enzymes. Furthermore, certain differences in the specific activities of the soluble and immobilized enzymes cannot be avoided, which may be caused by differences in the accessibility of the active sites to the substrate, small conformational changes, or modifications of the microenvironment. Diffusional limitation influencing the observed enzyme activity was presumed to be negligible due to the low protein loading. Finally, the mechanisms leading to irreversibility of inactivation may differ between soluble and immobilized enzymes, since autoproteolysis is widely prevented in carrier-bound enzymes. In both cases, the first unfolding step was assumed to be rate-limiting for inactivation.

Despite the limitations discussed above, the results of the present study provide a picture that is consistent with previous studies on the stability of TLP-ste as well as with our hypothesis concerning the stabilizing effect of immobilization. Immobilization was clearly most effective for the two specifically designed mutants (T56C, S65C) which have their cysteines within the postulated unfolding region of TLP-ste (*6*, *12*, *13*) (Figure 1**)**. The importance of residues 56 and 65 is supported by the more than 30-fold stabilization obtained upon immobilizing the T56C/S65C double mutant enzyme.

In contrast, immobilization of N181C, S218C, and T299C mutant enzymes, in which the cysteine residues are located far away from the postulated unfolding region (Figure 1), yielded much lower stabilization than immobilization via T56C and S65C. Obviously, the initial unfolding step is less hindered by fixation of these structural sites, which results in lower stabilization. Of course, it cannot be excluded that beneficial effects of immobilization might be offset by strain introduced into the TLP-ste structure in these mutants. However, modeling studies did not provide any reason to assume that N181C, S218C, and T299C are structurally less suitable for immobilization than T56C and S65C. The cysteines are fully solvent-exposed in all cases (Figure 1). This is also in accordance with the result that there were no systematic differences between these five mutants in terms of coupling yields or in the specific activities (Table 1).

Previously, it was shown that TLP-ste can be stabilized drastically by introducing a disulfide bond between residues 8 and 60, which connects the unfolding region with an underlying β-hairpin *(12)*. The corresponding single mutants G8C and N60C also used in this study were not a priori considered to be good candidates for immobilization, because in both cases the introduction of cysteine as well as subsequent coupling to the carrier were expected to have negative structural effects. For the G8C mutant, steric hindrance between Cys8 and Asn60 was anticipated. The removal of Asn60 in the N60C mutant enzyme without concomitant compensatory effects such as disulfide formation in the G8C/N60C mutant enzyme *(12)* was anticipated to be destabilizing because of the removal of a strong hydrogen bond with Thr22. Since the side chain of residue 60 is not fully solvent-exposed, immobilization was not expected to compensate for the negative effects of the N60C mutation. It is important to note that Asn60 is located next to Asp59, which is one of the two aspartate residues coordinating a calcium ion whose binding is known to contribute significantly to thermal stability of TLPs (*49*). As expected on the basis of these structural considerations, immobilization did not contribute to the stability of the G8C and N60C mutant enzymes. Also, the stabilizing effect of immobilizing the N60C/N181C double mutant enzyme was small and similar to that of the N181C single mutant enzyme.

In summary, the results demonstrate that the stabilizing effect of immobilization can be rationalized, once sufficient knowledge about the inactivation mechanism of the protein in question is available. From the studies on TLP-ste, combining site-specific immobilization with knowledge of the structural region where the unfolding process is initiated, it can be concluded that stabilization is most effective if the unfolding region is fixed, whereas it is much less effective if other regions are attached. In general, the localization of structural regions connected with early unfolding events should be the basis for rational strategies of protein stabilization, at least for proteins being inactivated irreversibly.

ACKNOWLEDGMENT

We thank Dr. Regina Schöps and Marion Sonntag for technical assistance. We thank Prof. G. Venema for providing stimulating working conditions and discussions.

REFERENCES

1. Ulbrich, R., Schellenberger, A., and Damerau, W. (1986) *Biotechnol. Bioeng. 28*, 511−522.
2. Schellenberger, A., and Ulbrich, R. (1989) *Biomed. Biochim. Acta 48*, 63−67.
3. Ulbrich-Hofmann, R., Golbik, R., and Damerau, W. (1993) in *Stability and Stabilization of Enzymes* (van den Tweel, W. J. J., Harder, A., and Buitelaar, R., Eds.) pp 497−504, Elsevier, London.
4. Damerau, W., Ulbrich, R., Lindig, C., and Schellenberger, A. (1984) *Stud. Biophys. 103*, 153−160.
5. Ulbrich-Hofmann, R., Mansfeld, J., Fittkau, S., and Damerau, W. (1995) *Biotechnol. Appl. Biochem. 22*, 75−94.
6. Vriend, G., and Eijsink, V. G. H. (1993) *J. Comput. Aided Mol. Des. 7*, 367−396.
7. Eijsink, V. G. H., Veltman, O. R., Aukema, W., Vriend, G., and Venema, G. (1995) *Nat. Struct. Biol. 2*, 374−379.
8. Braxton, S., and Wells, J. A. (1992) *Biochemistry 31*, 7796−7801.
9. Dahlquist, F. W., Long, J. W., and Bigbee, W. L. (1976) *Biochemistry 15*, 1103−111.
10. Van den Burg, B., Eijsink, V. G. H., Stulp, B. K., and Venema, G. (1990) *Biochem. J. 272*, 93−97.
11. Kidokoro, S.-I., Miki, Y., Endo, K., Wada, A., Nagao, H., Miyake, T., Aoyama, A., Yoneya, T., Kai, K., and Ooe, S. (1995) *FEBS Lett. 367*, 73−76.
12. Mansfeld, J., Vriend, G., Dijkstra, B. W., Veltman, O. R., Van den Burg, B., Venema, G., Ulbrich-Hofmann, R., and Eijsink, V. G. H. (1997) *J. Biol. Chem. 272*, 11152−11156.

13. Vriend, G., Berendsen, H. J. C., Van den Burg, B., Venema, G., and Eijsink, V. G. H. (1998) *J. Biol. Chem. 273*, 35074−35077.
14. Engen, J. R., Smithgall, T. E., Gmeiner, W. H., and Smith, D. L. (1997) *Biochemistry 36*, 14384−14391.
15. Ishii, Y. (1994) *Eur. J. Biochem. 221*, 705−712.
16. Arnold, U., Rücknagel, K. P., Schierhorn, A., and Ulbrich-Hofmann, R. (1996) *Eur. J. Biochem. 237*, 862−869.
17. Arnold, U., Schierhorn, A., and Ulbrich-Hofmann, R. (1998) *J. Protein Chem. 17*, 397−405.
18. Chrunyk, B. A., and Wetzel, R. (1993) *Protein Eng. 6*, 733−738.
19. Fontana, A., Polverino de Laureto, P., De Filippis, V., Scaramella, E., and Zambonin, M. (1997) *Fold. Des. 2*, R17−R26.
20. Ljungquist, C., Jansson, B., Moks, T., and Uhlén, M. (1989) *Eur. J. Biochem. 186*, 557−561.
21. Persson, M., Bülow, L., and Mosbach, K. (1990) *FEBS Lett. 270*, 41−44.
22. Iwakura, M., and Kokubu, T. (1993) *J. Biochem. 114*, 339−343.
23. McLean, M. A., Stayton, P. S., and Sligar, S. G. (1993) *Anal. Chem. 65*, 2676−2678.
24. Lu, B., Xie, J., Lu, C., Wu, C., and Wie, Y. (1995) *Anal. Chem. 67*, 83−87.
25. Huang, W., Wang, J., Bhattacharyya, D., and Bachas, L. G. (1997) *Anal. Chem. 69*, 4601−4607.
26. Nadkarni, V. D., Pervin, A., and Linhardt, R. J. (1994) *Anal. Biochem. 222*, 59−67.
27. Sigal, G. B., Bamdad, C., Barberis, A., Strominger, J., and Whitesides, G. M. (1996) *Anal. Chem. 68*, 490−497.
28. Nock, S., Spudich, J. A., and Wagner, P. (1997) *FEBS Lett. 414*, 223−238.
29. Kortt, A. A., Oddie, G. W., Iliades, P., Gruen, L. C., and Hudson, P. J. (1997) *Anal. Biochem. 253*, 103−111.
30. Iwane, A. H., Kitamura, K., Tokunaga, M., and Yanagida, T. (1997) *Biochem. Biophys. Res. Commun. 230*, 76−80.
31. Salamon, Z., Wang, Y., Tollin, G., and McLeod, H. A. (1994) *Biochim. Biophys. Acta 1195*, 267−275.
32. Eijsink, V. G. H., Dijkstra, B. W., Vriend, G., van der Zee, J. R., Veltman, O. R., van der Vinne, B., van den Burg, B., Kempe, S., and Venema, G. (1992) *Protein Eng. 5*, 421−426.
33. Eijsink, V. G. H., Vriend, G., van den Burg, B., Venema, G., and Stulp, B. K. (1990) *Protein Eng. 4*, 99−104.
34. Zell, R., and Fritz, H.-J. (1987) *EMBO J. 6*, 1809−1815.
35. Stanssens, P., Opsomer, C., McKeon, Y. M., Kramer, W., Zabeau, M., and Fritz, H.-J. (1989) *Nucleic Acids Res. 17*, 4441−4454.
36. Vriend, G. J. (1990) *J. Mol. Graphics* 8, 52−56.
37. Titani, K., Hermodson, M. A., Ericsson, L. H., Walsh, K. A., and Neurath, H. (1972) *Nature 238*, 35−37.
38. Barik, S., and Galinski, M. (1991) *BioTechniques 10*, 489−490.
39. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 643−657.
40. Van den Burg, B., Eijsink, V. G. H., Stulp, B. K., and Venema, G. (1989) *J. Biochem. Biophys. Methods 18*, 209−220.
41. Fujii, M., Takagi, M., Imanaka, T., and Aiba, S. (1983) *J. Bacteriol. 154*, 831−837.
42. Kunitz, M. (1947) *J. Gen. Physiol. 30*, 291−299.
43. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
44. Matthews, B. W., Jansonius, J. N., Colman, P. M., Schoenborn, B. P., and Dupourque, D. (1972) *Nature 238*, 37−41.
45. Holmes, M. A., and Matthews, B. W. (1982) *J. Mol. Biol. 160*, 623−639.
46. Veltman, O. R., Vriend, G., Hardy, F., Mansfeld, J., Van den Burg, B., Venema, G., and Eijsink, V. G. H. (1997) *Eur. J. Biochem. 248*, 433−440.
47. Imanaka, T., Shibazaki, M., and Takagi, M. (1986) *Nature 324*, 695−697.
48. Perry, L. J., and Wetzel, R. (1987) *Protein Eng. 1*, 101−195.
49. Veltman, O. R., Vriend, G., Berendsen, H. J. C., van den Burg, B., Venema, G., and Eijsink, V. G. H. (1998) *Biochemistry 37*, 5312−5319.

BI990008P