

# Single-point amino acid substitutions at the 119th residue of thermolysin and their pressure-induced activation

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**Abstract** The effect of amino acid substitution at the 119th site of thermolysin (TLN) on the pressure activation behavior of this enzyme was studied for four mutants at pressures < 300 MPa. For Q119Q, Q119N and Q119R, the highest activation was observed to be over 30 times that at atmospheric pressure and the activation volumes ( $\Delta V^\ddagger$ ) were about  $-75$  ml/mol. However, we obtained only 10 times higher activation for Q119E and Q119D ( $\Delta V^\ddagger \sim -60$  ml/mol). The intrinsic fluorescence of TLN changed at pressures > 300 MPa, and the latter two mutants showed a smaller  $\Delta G_{app}$  and  $\Delta V_{app}$  of transition than the wild type. These results are discussed with respect to the hydration change in the enzyme protein around the substituted region.

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**Key words:** Thermolysin; Pressure; Activation; Amino acid substitution; Protein stability

## 1. Introduction

Thermolysin (TLN) is a thermostable microbial neutral protease containing zinc as a cofactor. It has been studied extensively for its catalytic properties and structural aspects, ever since its primary and tertiary structures were elucidated [1,2], for both fundamental studies and bioengineering applications [3–6]. We have found that TLN showed a dramatic activation under moderately high hydrostatic pressure [7,8], about 100–200 MPa, and that this property could be exploited for the selective proteolysis of proteins and peptide condensations under high pressure [9–11]. In general, studies on enzymatic reactions and enzyme structure under high pressure have become a powerful tool in many fields [12–17].

The site-specific mutation of proteins and enzymes is a useful technique to study their structure and activity. Even a

mutation in a single amino acid residue not directly involved in the catalytic process may cause a large change in catalytic activity, and can result in a dramatic change in the structure and stability. Most of these mutational studies, however, have been performed in the context of the thermal behavior of the enzyme [18–22], i.e. high catalytic activity at high or very low temperatures and/or a stable structure under extreme temperatures. Studies on the effects of amino acid replacement on the pressure properties of enzymes and proteins, barostability and activity at higher pressure, have been rather few. The known examples include a nuclease from *Staphylococcae* [23–25] and a ribonuclease from *Sulfolobus solfataricus* [26,27]. In the former case, a paradoxical stabilization of the protein was produced by replacing the proline residues with glycine, and the effect of a replacement of F31 in the hydrophobic core of the latter thermostable enzyme was elucidated.

There have been several mutational studies on TLN and its homologous enzymes which elucidated their catalytic mechanisms and protein thermal stability (e.g. [28–35]). Kidokoro et al. prepared several sets of mutated TLN [31,35], and indicated that a replacement of the 143rd residue caused a large effect in the enzyme reaction mechanism, and that a substitution at the 119th residue profoundly affected its thermal stability and catalytic activity at higher temperature. Although the latter site was originally assigned as glutamic acid, recent cDNA sequencing [36] and a reassignment of the protein sequence [37] revealed that it was actually glutamine. Seventeen Q119 mutants were shown to exhibit an approximately linear and negative correlation between their thermal stability, as measured by differential calorimetry, and their catalytic activity toward a synthetic peptide substrate [31].

In the present study, we measured the enzyme activity of Q119 mutants of TLN under high pressure, especially with respect to their apparent pressure activation, and their stability against higher pressure as measured by changes in intrinsic fluorescence. Among the 17 Q119 mutants of TLN previously prepared [31], four mutants (Q119N, Q119R, Q119E and Q119D) and the wild type (Q119Q) were submitted for study. Three of them (Q119N, Q119D, and Q119Q) could be modelled on the above mentioned negative correlation between their activities and thermal stabilities, whereas Q119R deviated downward and Q119E deviated upward from this correlation line. Thus, Q119E showed the highest catalytic activity among the prepared mutants and Q119Q showed the lowest, whereas Q119Q was the most thermostable and Q119R was the most thermolabile.

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**Abbreviations:** Fua-, *N*-(3-[2-furyl]acryloyl)-; TLN, thermolysin; mut-TLNs, mutated thermolysins; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid

## 2. Materials and methods

### 2.1. Materials

The TLN mutants (mut-TLNs; Q119N, Q119R, Q119E and Q119D) were prepared as previously reported [31]. The concentration of the active enzyme was determined by a kinetic assay under our standard conditions (pH 6.2 and 25°C [38]) against a dipeptide substrates, *N*-(3-[2-furyl]acryloyl)-glycyl-L-leucine amide (Fua-Gly-LeuNH<sub>2</sub>), which was purchased from Bachem (Switzerland). The other dipeptide substrates used for the specificity study, Fua-Asp-PheNH<sub>2</sub>, Fua-Gly-PheNH<sub>2</sub>, Fua-Phe-PheNH<sub>2</sub>, Fua-Phe-GlyNH<sub>2</sub> and Fua-Phe-ValNH<sub>2</sub>, and the tripeptide substrate, Fua-Gly-Leu-Ala, were synthesized by the hydroxysuccinimide method [39] or purchased from Bachem. The buffer reagent (2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; HEPES) was obtained from Dojindo Laboratories (Kumamoto, Japan). All other chemicals were purchased from Nacalai Tesque (Kyoto, Japan).

### 2.2. Methods

The kinetic assay under atmospheric pressure was performed with a spectrophotometer UV2200 (Shimadzu, Kyoto, Japan). The absorbance changes under elevated pressure were measured in a high pressure optical cell made by Teramecs (Kyoto, Japan), which was connected to a deuterium lamp and detector (PRAS-5000, Otsuka Electronics, Hirakata, Japan) through optical fibers. The temperature of the sample solution in the cell was controlled by a Peltier-type thermoregulator and detected by a Pt resistance thermometer. The fluorescence under ambient and high pressures was monitored in an optical high pressure vessel with three sapphire windows and a quartz inner optical cell (Teramecs, Kyoto, Japan). The vessel was located in the sample chamber of a spectrofluorometer RF5000 (Shimadzu, Kyoto, Japan) with thermostated water circulating through the cell block. The temperature of the water inside the vessel, just outside the inner cell, was detected by a Cu-constantan thermocouple. In both cases, external pressure was applied by a high pressure hand pump equipped with an intensifier (ratio 8.5:1) (Teramecs); the pressure medium was deionized water. The pressure was measured by a Bourdon tube-type pressure gauge.

## 3. Results

### 3.1. Specificity of mut-TLNs for synthetic substrates

The apparent second-order rate constants ( $k_{\text{cat}}/K_{\text{m(app)}}$ ) of the mut-TLNs were evaluated at atmospheric pressure for seven synthetic peptide substrates using relatively low substrate concentrations (<0.6 mM). The values relative to the constant for Fua-Gly-LeuNH<sub>2</sub> ( $2.0 \times 10^4 \text{ s}^{-1} \text{ M}^{-1}$  at pH 6.5 and 37°C) are shown in Fig. 1 as relative specificity. All enzymes showed the highest specificity towards Fua-Phe-PheNH<sub>2</sub>; Fua-Phe-ValNH<sub>2</sub> was the second and Fua-Gly-Leu-Ala was the third best, among the tested substrates. The highest rate constant for each substrate was always observed for Q119E. However, the discrimination at the P<sub>1</sub> position was highest in Q119R (Phe/Gly = ca. 680) and the highest at the P<sub>1</sub>' position was observed in Q119N for Leu/Phe (= ca. 2) or in Q119R for Phe/Val (ca. 9). No enzymes reacted with Fua-Phe-GlyNH<sub>2</sub>; in this case, the specificity value for

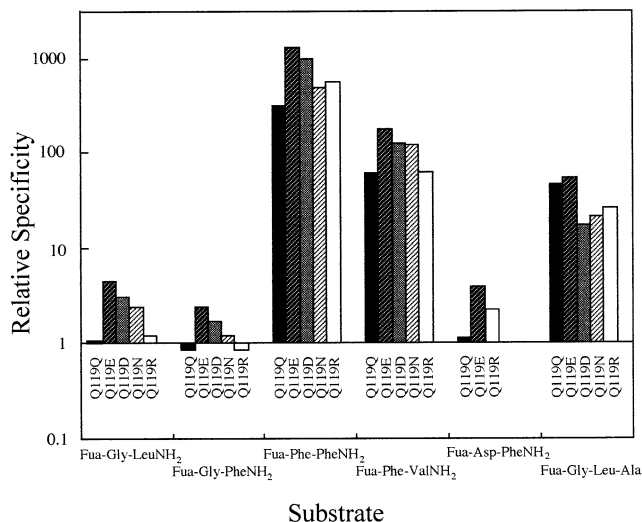


Fig. 1. Relative specificity of TLN mutants at the 119th site for six peptide substrates. The apparent second-order rate constant for each substrate and each mutant is shown as the value relative to that for Fua-Gly-LeuNH<sub>2</sub> with the wild type. At 37°C, 0.1 MPa, and pH 6.5 (50 mM HEPES-NaOH). [substrate] < 0.6 mM, [enzyme] = 4–45 nM, and [CaCl<sub>2</sub>] = 10 mM.

Phe/Gly at the P<sub>1</sub>' position was very large ( $\geq 10^3$ ), and this substrate practically acted as an inhibitor for all of the tested enzymes.

### 3.2. Hydrolytic activity of mut-TLNs under high pressure

The hydrolytic activities ( $k_{\text{cat}}/K_{\text{m(app)}}$ ) for Fua-Gly-LeuNH<sub>2</sub> of mut-TLNs and the wild type were measured at various pressures, and the results are shown in Fig. 2. In all cases, the elevation of pressure up to ca. 200 MPa increased the reaction rate, as was reported for the wild type enzyme previously [7,8]. However, the extent of the pressure-induced activation was dependent on the mutated amino acid residue. The maximum rate for Q119Q, Q119N and Q119R reached about 30–40 times that of the rate at atmospheric pressure, but increased only about 10-fold for Q119E and Q119D. The pressure value showing the apparent maximum rate ( $P_{\text{max}}$ ) varied, and shifted to the low pressure side for some mutants. The  $P_{\text{max}}$  was approximately 240 MPa for Q119Q, Q119N and Q119R, but was around 180 MPa for Q119E and Q119D. Since the hydrolytic activity of these mutant enzymes towards the substrate differed as mentioned above, the highest apparent rate was observed at 240 MPa for Q119N.

In general, pressure-dependent events, rate constants and equilibrium constants at constant temperature, can be analyzed by an equation composed of the first and second derivatives of the free energy with respect to pressure. In the case of the rate constant ( $k$ ), as in the present study, the first derivative is interpreted as the volume of activation ( $\Delta V^{\ddagger}$ ) and the second derivative as the (absolute) compressibility of activation ( $\Delta\beta^{\ddagger}$ ). In Eq. 1, the subscript o means the standard condition (0.1 MPa). Please note that this (absolute) compressibility takes on a negative value when the volume decreases with pressure, while the conventional relative compressibility takes on a positive value with decreasing volume.

$$\ln k = \ln k_o - ((\Delta V^{\ddagger}/RT) \cdot P + (\Delta\beta^{\ddagger}/2RT) \cdot P^2) \quad (1)$$

Table 1

The activation volumes and absolute compressibility of activation calculated for the specificity factor ( $k_{\text{cat}}/K_{\text{m}}$ ) of the TLN-catalyzed hydrolysis of Fua-Gly-LeuNH<sub>2</sub> at 25°C

Mutant	$\Delta V_{\text{app}}^{\ddagger}$ (ml/mol)	$\Delta\beta_{\text{app}}^{\ddagger}$ ( $10^{-1}$ ml/mol/MPa)
Q119Q	-74	-2.6
Q119N	-74	-3.5
Q119R	-77	-2.5
Q119E	-59	-3.4
Q119D	-56	-3.0

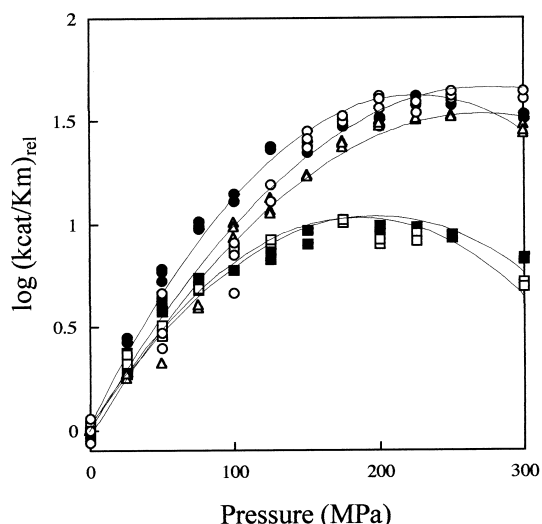


Fig. 2. Pressure dependence of the apparent second-order rate constant of the hydrolysis by wild-type TLN and mutants thereof.  $\circ$ , Q119Q;  $\bullet$ , Q119N;  $\triangle$ , Q119R;  $\square$ , Q119E;  $\blacksquare$ , Q119D. At 37°C and pH 6.5 (50 mM HEPES-NaOH). [substrate] < 0.6 mM, [enzyme] = 4–45 nM, and [CaCl<sub>2</sub>] = 10 mM.

The values of  $\Delta V_{app}^{\ddagger}$  and  $\Delta\beta_{app}^{\ddagger}$  were calculated from the data in Fig. 2 by curve fitting to Eq. 1, and are compiled in Table 1. It is also to be noted that these parameters (especially  $\Delta\beta_{app}^{\ddagger}$ ) are calculated from the obtained apparent rates and only apparent parameters, since the negative  $\Delta\beta^{\ddagger}$  values originate, as explained below, from the pressure-induced irreversible deactivation of the enzyme.

Q119Q, Q119N and Q119R showed large values of activation volume, whereas Q119E and Q119D had smaller values. It is assumed that the comparatively large  $\Delta V_{app}^{\ddagger}$  values of TLN are due to a change in the hydration state of the enzyme molecule coupled with the progress of the reaction [7]; the number of electrostricted water molecules with a smaller partial molar volume is increased at the transition state of the catalytic reaction.

The pressure dependence of  $k_{cat}$  and  $K_m$  towards Fua-Asp-PheNH<sub>2</sub> was measured by changing the substrate concentration up to 0.1 mM for Q119E, Q119R, and the wild type. The resultant values ( $\Delta V_{kcat}^{\ddagger}$  and  $-\Delta V_{Km}$ ; the minus sign of the latter corresponds to the direction of the volume change in the reaction progress) were as follows;  $\Delta V_{kcat}^{\ddagger} = -50$  ml/mol and  $-\Delta V_{Km} = -10$  ml/mol for the wild type,  $\Delta V_{kcat}^{\ddagger} = -28$  ml/mol and  $-\Delta V_{Km} = -5$  ml/mol for Q119E, and  $\Delta V_{kcat}^{\ddagger} = -50$  ml/mol and  $-\Delta V_{Km} = -5$  ml/mol for Q119R. Both volume parameters became smaller by changing the Q to E at the 119th site, reflecting the observed smaller pressure activation, but the  $k_{cat}$  factor became relatively important.

Table 2

The apparent transition volume and the Gibbs free energy of fluorescence intensity change for five TLNs, compared with the thermal stability

Enzyme	$\Delta V_{app}$ (ml/mol)	$\Delta G_{app}$ (kJ/mol)	$T_p^a$ (°C)
Wild type	-75	25	89
Q119N	N.D. <sup>b</sup>	N.D.	85
Q119R	-134	40	81
Q119E	-38	17	82
Q119D	-35	12	81

<sup>a</sup>Transition temperature determined by the DSC method at atmospheric pressure. Taken from [31].

<sup>b</sup>Not determined.

### 3.3. The fluorescence change of TLNs under hydrostatic pressure

The small but distinct variation in the compressibility of activation, together with variable pressure value giving the apparent maximum activation, led us to measure the change in the intrinsic fluorescence (mostly from the three tryptophan residues) of the mut-TLNs under increasing pressure. Fig. 3 summarizes the results in the form of relative fluorescence peak intensity changes (a) and peak wavelength (b); both the intensity and the peak wavelength showed deviation at a higher pressure. The decrease in fluorescence intensity and the red shift of the peak both indicate that the environment around the tryptophan residues became more polar. Although these changes are the average of the sum of the changes in all three Trp residues in TLN, it may be postulated that the enzymatic structure gradually collapses under increasing pressure, and that these aromatic residues become more accessible for the solvent water. In the case of the wild type, the distinct change was seen at over 300 MPa, as was observed by the fourth derivative spectrum measurement, but some mutant proteins showed a rather small or vague transition. The intensity change was analyzed by assuming a two-state transition, and the apparent Gibbs free energy change ( $\Delta G_{app}$ ) and volume change ( $\Delta V_{app}$ ) of the transition were calculated by curve fitting to Eq. 2.

$$F(P) = F1 + (F2 - F1) / [1 + \exp\{-(\Delta G_{app} + P \cdot \Delta V_{app}) / (RT)\}], \quad (2)$$

where  $F1$  and  $F2$  correspond to the fluorescence intensity of the first (low pressure) state and the second (high pressure) state of the protein, respectively, and  $F(P)$  is the observed fluorescence intensity at pressure  $P$ . Table 2 shows the results. When compared with the values from the wild type, the two substituted mutants with residues having a negative charge (Q119E and Q119D) showed almost half of the normal values for both  $\Delta V_{app}$  and  $\Delta G_{app}$ . The Q119R enzyme showed very large values for both parameters, but the second (high pressure) state of the protein seems to have a relatively high fluorescence intensity, although the peak shift was almost comparable to that of the wild type.

## 4. Discussion

The higher activity of mut-TLNs, compared with wild type, under atmospheric pressure can be explained as follows [31]: the 119th residue fixes the strand from residues 112–117 to other parts of the protein domain by forming (a) hydrogen bond(s) and the breakage of this hydrogen bond or these bonds by replacement of the 119th amino acid residue increases the strand flexibility, in order to facilitate the binding

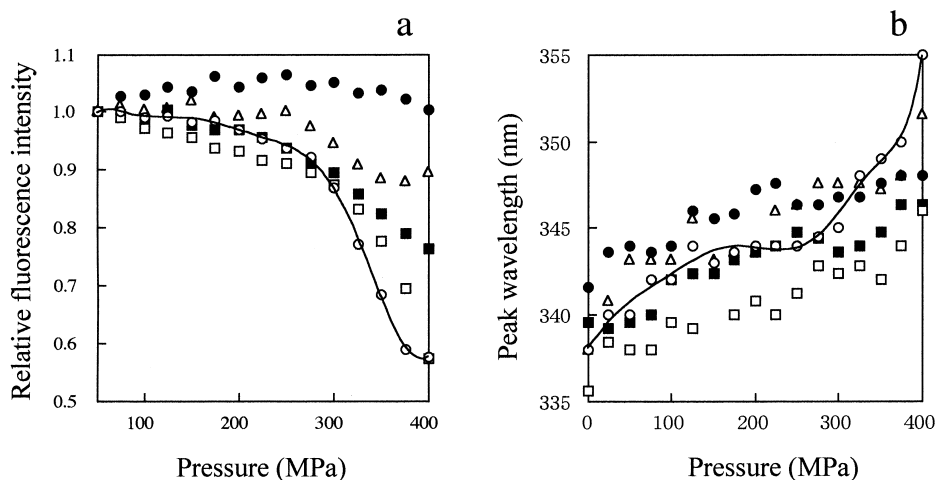


Fig. 3. The fluorescence changes of TLN solutions under high pressure. a: The relative change in the peak intensity. b: The shift in the peak wavelength.  $\circ$ , Q119Q;  $\bullet$ , Q119N;  $\triangle$ , Q119R;  $\square$ , Q119E;  $\blacksquare$ , Q119D. At 37°C and pH 6.5 (50 mM HEPES-NaOH). [enzyme]=70–90 nM and  $[\text{CaCl}_2]=10$  mM.  $\lambda_{\text{ex}}=280$  nm. The solid curve was fitted to the points of the wild type by assuming a two-state transition model (Eq. 2).

of the substrate to the enzyme and to increase the activity, since most of the effects were observed in the  $K_m$  parameter.

The large and negative activation volume, or in other words the apparent pressure-induced activation, of TLN cannot be explained solely by factors in the catalytic sites, but is also related to structural changes coupled with the progress of the reaction [40]. The hinge-bending motion during catalysis between the two distinct domains of TLN, as proposed by Holland et al. [34,41,42], would take part in such a concerted structural motion. These changes in the structure or structural motions may result in an increase in hydration during the transition state, which is reflected by the activation volume, and thus favors a high pressure environment. In other words, the magnitude of the pressure-induced activation is determined by the hydration difference between the ground state and the transition state of the reaction. Furthermore, this increased hydration may have already occurred during the non-bonded complex formation between the enzyme and the substrate (usually presented as the E·S complex) in the wild type enzyme, and hence the  $-\Delta V_{K_m}$  value becomes large and negative.

By introducing a negatively charged amino acid residue at the 119th position, the hydration of the ground state will be increased as compared with the wild type. This substitution also increases the catalytic activity under atmospheric pressure. However, the relative increase in hydration at the transition state might become smaller by this hydration increase at the ground state, in order to decrease the extent of the pressure-induced activation, as a result. The hydration change promoting the reaction is countered by an inactivation of the enzyme at higher pressure, and this cancels out the increased activity induced by higher pressure. Therefore, this results in an apparent maximum in the pressure dependence of the activity. Over this pressure range, the fluorescence changes become evident, and it apparently obeys a simple two-state transition model with the midpoint pressure at approximately 350 MPa for the wild type. The volume change for this transition is  $-75$  ml/mol for the wild type, but becomes halved upon replacement with a negatively charged residue. These negatively charged mutants show some pressure tolerance, although they lose their thermal stability on

the other hand [31]. The pressure value giving the apparent maximum ( $P_{\text{max}}$ ), and hence the apparent values of  $\Delta\beta^\ddagger$ , will be determined by a balance between the extent of the pressure activation and the pressure stability of the enzyme protein. The apparently lower  $P_{\text{max}}$  values and the larger values of  $\Delta\beta^\ddagger$  observed for Q119D and Q119E will be mainly related to the lower pressure-induced activation of these two mutants, even with the increased pressure stability as observed by fluorimetry.

## References

- [1] Titani, K., Hermodson, M.A., Ericsson, L.H., Walsh, K.A. and Neurath, H. (1972) *Nature New Biol.* 238, 35–37.
- [2] Matthews, B.W., Jansonius, P.M., Colman, B.P., Schoenborn, B.P. and Dupor, D. (1972) *Nature New Biol.* 238, 37–41.
- [3] Morgan, G. and Fruton, J.S. (1978) *Biochemistry* 17, 3562–3568.
- [4] Isowa, Y., Ohmori, M., Ichikawa, T. and Mori, K. (1979) *Tetrahedron Lett.* 28, 2611–2612.
- [5] Wayne, S.I. and Fruton, J.S. (1983) *Proc. Natl. Acad. Sci. USA* 80, 3241–3244.
- [6] Morihara, K. (1987) *Trends Biotechnol.* 5, 164–170.
- [7] Fukuda, M. and Kunugi, S. (1984) *Eur. J. Biochem.* 142, 565–570.
- [8] Kunugi, S., Kitayaki, M., Yanagi, Y., Tanaka, N., Lange, R. and Balny, C. (1997) *Eur. J. Biochem.* 248, 567–574.
- [9] Kunugi, S., Tanabe, K., Yamashita, K., Morikawa, Y., Ito, T., Kondoh, T., Hirata, K. and Nomura, A. (1989) *Bull. Chem. Soc. Jpn.* 62, 514–518.
- [10] Hayashi, R., Kawamura, Y. and Kunugi, S. (1987) *J. Food Sci.* 52, 1107–1108.
- [11] Kunugi, S., Kanazawa, Y., Mano, K., Koyasu, A. and Inagaki, T. (1996) in: *Progress in Biotechnology 13* (Hayashi, R. and Balny, C., Eds.), pp. 178–189, Elsevier, Amsterdam.
- [12] Hayashi, R. and Balny, C. (1996) in: *Progress in Biotechnology 13* (Hayashi, R. and Balny, C., Eds.), Elsevier, Amsterdam.
- [13] Heremans, K. (1997) *High Pressure Research in the Biosciences and Biotechnology*, Leuven University Press, Leuven.
- [14] Ludwig, H. (1999) *High Pressure Bioscience and Biotechnology*, R.-K.-Universität Heidelberg Verlag, Heidelberg.
- [15] Gross, M. and Jaenicke, R. (1994) *Eur. J. Biochem.* 221, 617–630.
- [16] Mozhaev, V.V., Heremans, K., Frank, J., Masson, P. and Balny, C. (1994) *Trends Biotechnol.* 12, 493–501.
- [17] Mozhaev, V.V., Heremans, K., Frank, J., Masson, P. and Balny, C. (1996) *Proteins Struct. Funct. Genet.* 24, 81–91.

- [18] Vieille, C., Burdette, D.S. and Zeikus, J.G. (1996) *Biotechnol. Annu. Rev.* 2, 1–83.
- [19] Russell, R.J. and Taylor, G.L. (1995) *Curr. Opin. Biotechnol.* 6, 370–374.
- [20] Vriend, G. and Eijsink, V. (1993) *J. Comput. Aided Design* 7, 367–396.
- [21] Nosoh, Y. and Sekiguchi, T. (1990) *Trends Biotechnol.* 8, 16–20.
- [22] Mozhaev, V.V. (1993) *Trends Biotechnol.* 11, 88–95.
- [23] Royer, C.A., Hinck, A.P., Loh, S.N., Prehoda, K.E., Peng, X.-D., Jonas, J. and Markley, J.L. (1993) *Biochemistry* 32, 5222–5232.
- [24] Vidugiris, G.J.A., Truckses, D.M., Markley, J.L. and Royer, C.A. (1996) *Biochemistry* 35, 3857–3864.
- [25] Frye, K.J., Perman, C.S. and Royer, C.A. (1996) *Biochemistry* 35, 10234–10239.
- [26] Mombelli, E., Afshar, M., Fusi, P., Mariani, M., Tortora, P., Connelly, J. and Lange, R. (1997) *Biochemistry* 36, 8733–8742.
- [27] Fusi, P., Goossens, K., Consonni, R., Puridelli, P., Vecchio, G., Vanonin, M., Zetta, L., Heremans, K. and Tortora, P. (1997) *Proteins Struct. Funct. Genet.* 29, 381–390.
- [28] Toma, S., Campagnoli, S., De Gregoriis, E., Gianna, R., Margarit, I., Zamai, M. and Grandi, G. (1989) *Protein Eng.* 2, 359–364.
- [29] Eijsink, V.G., Vriend, G., Van den Burg, B., Venema, G. and Stulp, B.K. (1990) *Protein Eng.* 4, 99–104.
- [30] Meaumont, A., O'Donohue, M.J., Paredes, N., Rousselet, N., Assicot, M., Bohuon, C., Fournie-Zaluski, M.C. and Roques, B.P. (1995) *J. Biol. Chem.* 270, 16803–16808.
- [31] Kidokoro, S., 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.
- [32] Frigerio, F., Margarit, T., Nogarotto, R., de Filippis, V. and Grandi, G. (1996) *Protein Eng.* 9, 439–445.
- [33] Mansfeld, J., Vriend, G., Dijkstra, B.W., Veltman, O.R., Van den Burg, B., Venema, G., Ulbrich-Hofmann, R. and Eijsink, V.G. (1997) *J. Biol. Chem.* 272, 11152–11156.
- [34] Veltman, O.R., Eijsink, V.G., Vriend, G., de Kreijl, A., Venema, G. and Van den Burg, B. (1998) *Biochemistry* 37, 5305–5311.
- [35] Kidokoro, S. (1998) *Adv. Biophys.* 35, 121–143.
- [36] O'donohue, J.M., Roques, B.P. and Bequimout, A. (1994) *Biochem. J.* 300, 599–603.
- [37] Miki, Y. (1994) *J. Ferm. Bioeng.* 77, 457–458.
- [38] Kunugi, S., Hirohara, H. and Ise, N. (1982) *Eur. J. Biochem.* 124, 157–163.
- [39] Blumberg, S. and Vallee, B.L. (1975) *Biochemistry* 14, 2410–2419.
- [40] Low, P.S. and Somero, G.N. (1975) *Proc. Natl. Acad. Sci. USA* 72, 3014–3018.
- [41] Holland, D.R., Tronrud, D.E., Pley, H.W., Flaherty, K.M., Stark, W., Jansonius, J.N., McKay, D.B. and Matthews, B.W. (1992) *Biochemistry* 31, 11310–11316.
- [42] van Aalten, D.M., Amadei, A., Linssen, A.B., Eijsink, V.G., Vriend, G. and Berendsen, H.J. (1995) *Proteins* 22, 45–54.