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Effect of freezing on the enzymatic coupling of specific amino acid-containing peptide fragments

Nicole Wehofsky,[a] Marion Haensler,[a] Sebastian W. Kirbach,[a] Johannes-Dieter Wissmann[a] and Frank Bordusa[a,b,*]

[a]*University of Leipzig, Department of Biochemistry, Talstr. 33, D-04103 Leipzig, Germany*
[b]*Max-Planck Society, Research Unit 'Enzymology of Protein Folding', Weinbergweg 22, D-06120 Halle, Germany*

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Abstract

The effect of freezing on the enzymatic coupling of highly specific amino acid-containing peptide fragments was investigated using trypsin, α-chymotrypsin, and *Bacillus licheniformis* Glu-specific endopeptidase as biocatalysts. Comparison with reactions at normal temperature indicates that freezing efficiently represses the cleavage of specific peptide bonds independent of their individual localisation and specificity achieving irreversible and efficient peptide bond formation without proteolytic side reactions.

1. Introduction

Proteases have long been known to catalyse CN-bond formations with high regio- and stereoselectivity under mild reaction conditions.[1–3] Thus, they reduce the need for protecting groups and typically give enantiomerically pure products. Several problems, however, still remain that hinder the wide acceptance of this enzymatic strategy for peptide synthesis. For the coupling of longer peptide fragments, undesired proteolysis of internal peptide bonds mediated by the enzyme is particularly troublesome. In addition, the enzyme catalyses the hydrolysis of the acyl donor ester simultaneously with the coupling reaction which inevitably results in a decrease of peptide product yield. A compromise solution for controlling these side reactions lies in the use of organic solvents as the medium of reaction.[4–6] It was found that a high content, for instance of dimethylformamide, dimethyl sulfoxide or acetonitrile, represses the rate of proteolytic cleavages of the peptide reactants or products as well as the competitive hydrolysis of the acyl donor ester to a certain extent. Unfortunately, many proteases suffer low activity and poor stability accompanied

* Corresponding author. Tel: +49 341 9736912; fax: +49 341 9736998; e-mail: bordusa@uni-leipzig.de

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by the permanent danger of enzyme deactivation in such solvents.[7] Another drawback to the use of pure organic solvents is that the destruction of enzyme enantioselectivity often occurs in such environments.[8,9] Also, when coupling large peptide fragments, the substrates are frequently poorly soluble in anhydrous media. Thus, a very careful and often time-consuming optimisation of reaction conditions is required that finally prevents the use of this strategy from being easily and universally applicable.

Freezing the reaction mixture appears to have similar beneficial effects on the enzymatic synthesis reaction.[10–14] It also decreases the rate of competitive hydrolysis of the acyl donor ester as well as of the newly formed peptide bonds application. In contrary, the derogation of the enzyme activity and stability is usually significantly lower. Although this effect of freezing could be verified for numerous proteases, up until now there are no systematic studies on the application of this approach to repress proteolytic side reactions at highly specific amino acid moieties within the peptide reactants. Since these cleavage sites often represent significantly more specific targets than the newly formed peptide bonds,[15] it is questionable whether freezing decreases the rate of their cleavage in the same manner than that of the newly formed peptide bonds. This knowledge, however, is essential for the general synthetic use of the freezing approach to the coupling of peptide segments.

In this paper we report on the application of the freezing approach to repress unwanted proteolytic reactions in protease-mediated couplings of specific amino acid-containing peptides. For this purpose, proteases with distinct specificities, i.e. trypsin, α-chymotrypsin, and *Bacillus licheniformis* Glu-specific endopeptidase (BL-GSE), have been selected as model enzymes and where used as biocatalysts for the coupling of pentapeptides bearing appropriate specific amino acid moieties at position 3. Comparison of the results under frozen state conditions with those at normal temperature indicates that freezing represents a useful strategy to control proteolytic side reactions within the starting peptide reactants and products as well as achieving high yields of intact peptide products.

2. Results and discussion

In enzymatic peptide synthesis the reactivity of the protease towards the acyl donor ester represents the key parameter that determines the extent of competitive proteolytic side reactions.[1] Generally, the higher the reactivity the lower the risk of unwanted proteolytic reactions during synthesis. Besides the individual C-terminal amino acid moiety, the ester leaving group of the acyl donor particularly affects the reactivity towards proteases.[16–18] For economic reasons, however, the use of simple alkyl esters, i.e. methyl or ethyl esters, is preferred over that of more specific fine-tuned and, thus, more expensive ones. Furthermore, it is known that the reactivity of proteases usually increases with an elongation of the peptide chain of the acyl donor.[15] Considering these facts, we selected simple methyl or ethyl esters of specific N^{α}-blocked amino acids as the acyl donor components. The individual amino acid moiety was chosen according to the specificity of the appropriate protease, i.e. Arg for trypsin, Tyr in the case of α-chymotrypsin, and Glu for BL-GSE. The courses of the enzymatic coupling of these acyl donors with appropriate specific amino acid-containing pentapeptides are illustrated in Figs. 1–3. On analysis of the enzymatic reactions at normal temperature (Figs. 1a–3a), in all cases proteolytic cleavages of the appropriate sensitive bonds within the nucleophilic peptides could be observed. In the case of trypsin and α-chymotrypsin, these cleavages already start before complete acyl donor ester consumption

and, thus, occur simultaneously to the coupling reactions. Contrary, BL-GSE attacks the sensitive Glu–Ala-bond within the former pentapeptide with lower rates and only after reaching the product optimum. This distinct behaviour may be attributed to a higher specificity of BL-GSE towards ester substrates relative to that towards peptidic ones as well as to the only low product yield which should compulsively lead to lower rates of peptide proteolysis. Contrary, the newly formed peptide bonds are generally less specific targets. Thus, cleavages at these positions do practically not affect the course of syntheses in all three cases. This observation confirms the fact that the position of the site-specific amino acid moiety significantly affects the extent of proteolytic side reactions. From a synthetic point of view, our results show that cleavages of the newly formed peptide bonds can be avoided by a simple time control of synthesis. The coupling of specific amino acid-containing fragments, however, essentially requires further efforts to minimise unwanted proteolytic side reactions during synthesis.

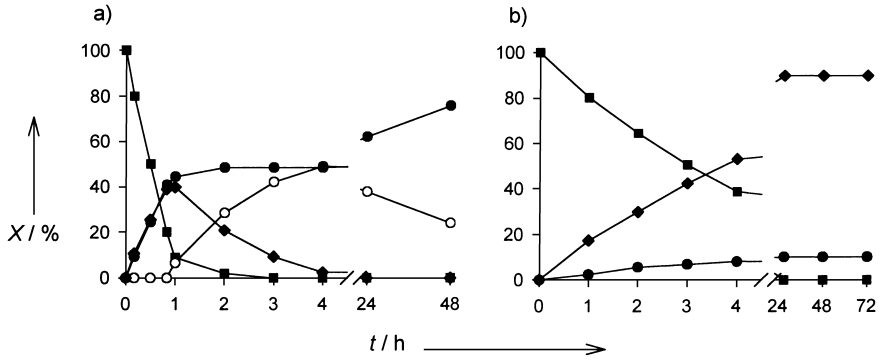

Figure 1. Course of the trypsin-catalysed coupling of Bz-Arg-OEt with H-Ala-Ala-Arg-Ala-Gly-OH. (a) 25°C; (b) –15°C. (–■–) Bz-Arg-OEt; (–◆–) Bz-Arg-Ala-Ala-Arg-Ala-Gly-OH; (–○–) Bz-Arg-Ala-Ala-Arg-OH; (–●–) Bz-Arg-OH. Conditions at 25°C: 0.1 M Hepes buffer (pH 8.0), 0.2 M NaCl, 0.02 M $CaCl_2$, [Bz-Arg-OEt] = 2 mM, [H-Ala-Ala-Arg-Ala-Gly-OH] = 15 mM, [trypsin] = 1.0×10^{-8} M. Conditions at –15°C: distilled water (pH 8.0 before freezing), [Bz-Arg-OEt] = 2 mM, [H-Ala-Ala-Arg-Ala-Gly-OH] = 15 mM, [trypsin] = 1.0×10^{-6} M

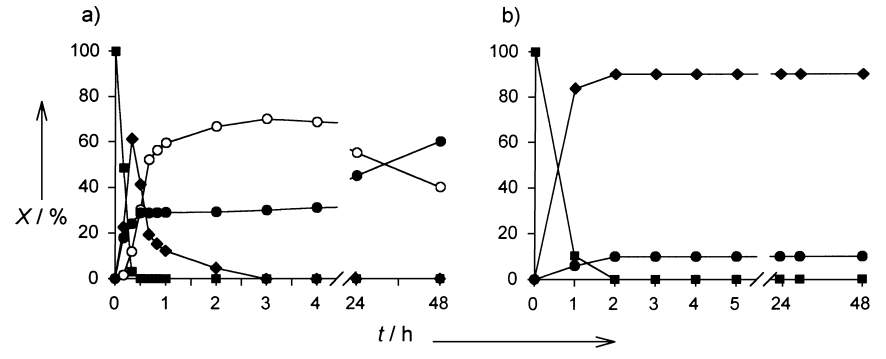

Figure 2. Course of the α-chymotrypsin-catalysed coupling of Ac-Tyr-OEt with H-Ala-Ala-Tyr-Ala-Gly-OH. (a) 25°C; (b) –15°C. (–■–) Ac-Tyr-OEt; (–◆–) Ac-Tyr-Ala-Ala-Tyr-Ala-Gly-OH; (–○–) Ac-Tyr-Ala-Ala-Tyr-OH; (–●–) Ac-Tyr-OH. Conditions at 25°C: 0.1 M Hepes buffer (pH 8.0), 0.2 M NaCl, 0.02 M $CaCl_2$, [Ac-Tyr-OEt] = 2 mM, [H-Ala-Ala-Tyr-Ala-Gly-OH] = 15 mM, [α-chymotrypsin] = 1.0×10^{-8} M. Conditions at –15°C: distilled water (pH 8.0 before freezing), [Ac-Tyr-OEt] = 2 mM, [H-Ala-Ala-Tyr-Ala-Gly-OH] = 15 mM, [α-chymotrypsin] = 1.0×10^{-7} M

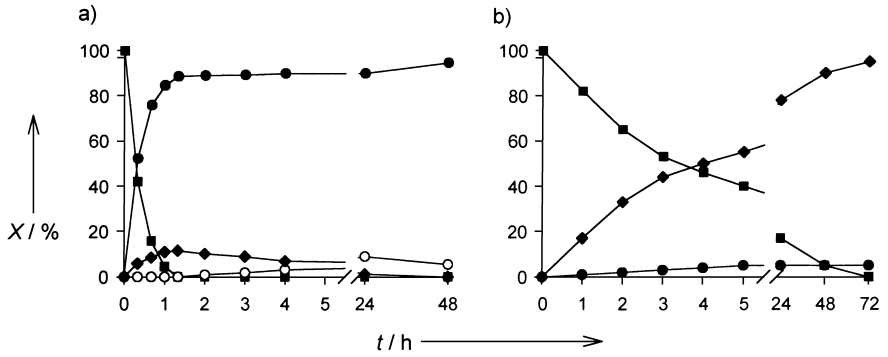

Figure 3. Course of the BL-GSE-catalysed coupling of Z-Glu-OMe with H-Ala-Ala-Glu-Ala-Gly-OH. (a) 25°C; (b) –15°C. (–■–) Z-Glu-OMe; (–◆–) Z-Glu-Ala-Ala-Glu-Ala-Gly-OH; (–○–) Z-Glu-Ala-Ala-Glu-OH; (–●–) Z-Glu-OH. Conditions at 25°C: 0.1 M Hepes buffer (pH 8.0), 0.2 M NaCl, 0.02 M $CaCl_2$, [Z-Glu-OMe] = 2 mM, [H-Ala-Ala-Glu-Ala-Gly-OH] = 15 mM, [BL-GSE] = 1.0×10^{-8} M. Conditions at –15°C: distilled water (pH 8.0 before freezing), [Z-Glu-OMe] = 2 mM, [H-Ala-Ala-Glu-Ala-Gly-OH] = 15 mM, [BL-GSE] = 1.0×10^{-8} M

To estimate the effect of freezing on such cleavages, similar reactions at sub zero temperature were performed. A reaction temperature of –15°C was proved to be a suitable compromise between activity and synthetic efficiency of most enzymes.[19] While the concentrations of the reactants correspond to those at room temperature, the enzyme concentrations were adjusted according to the reduced enzyme activities under these conditions. The use of buffer was avoided because of the unpredictable changes in pH in frozen aqueous systems. Plots of the enzymatic coupling reactions are illustrated in Figs. 1b–3b. Comparison of the reactions at –15°C with those at room temperature indicates that freezing has two main effects on the course of synthesis: (i) increase of aminolysis rates and, thus, increase of product yields; (ii) repression of proteolytic cleavages of the newly formed peptide bonds as well as after specific amino acid moieties within the former nucleophilic peptides. The latter indicates that the effect of freezing on the proteolytic stability of peptide bonds seems to be independent of the position and, thus, of the specificity of the protease towards the individual cleavage site. From a synthetic point of view, this offers practical irreversible peptide bond formation without the risk of unwanted proteolytic reactions within the peptide reactants as well as products. The generally lower rates of enzymatic reactions in the frozen system can be easily compensated either by the elongation of the reaction time or by a further increase of enzyme concentration. Generally, due to the high stability of the reactants under frozen conditions, longer incubation times do not favour the formation of non-enzymatic hydrolysis or aminolysis by-products as indicated by the course of syntheses (cf. Figs. 1b–3b). The higher acylation rates under frozen state conditions indicate that freezing not only represses the cleavage of specific peptide bonds, but simultaneously the hydrolysis of the acyl donor esters. As a general reason for this yield-increasing effect the 'freeze–concentration model' has been postulated.[20] According to this, freezing the reaction mixture results in a drastic increase in concentration of all reactants in the remaining unfrozen liquid phase which finally raises the efficiency of coupling. Most remarkably, in the case of BL-GSE this concentration effect increases the yield of intact peptide product from about 10% at room temperature to approximately 95% at –15°C. In the case of trypsin- and α-chymotrypsin-catalysed syntheses, the product yields increase by a factor of about 2 and 1.5, respectively, reaching yields of about 90% under frozen conditions. Comparison of these results with those found for the coupling of nucleophilic dipeptides without site-specific amino acid moieties (Table 1) does not reveal significant differences. This indicates

that the effect of freezing appears to be independent of the presence of additional highly specific cleavage sites within the reactants or the resulting products. Reactions using other specific amino acid-containing peptides such as Lys in the case of trypsin, Phe for α-chymotrypsin, and Asp for BL-GSE confirm these results indicating that these freezing-effects on the efficiency of synthesis and the stability of specific cleavage sites could be generalised.

Table 1
Influence of freezing on the coupling of specific amino acid-containing peptides catalysed by trypsin, α-chymotrypsin, and BL-GSE. The specific amino acid moieties within the nucleophilic peptides are underlined. Brackets generally indicate competitive cleavages. The data in brackets correspond to the whole peptide products while the data outside gives the yields of the appropriate intact hexapeptide product[a]

Protease / Acyl donor	Acyl acceptor	Yield [%]	
		25 °C	–15 °C
trypsin			
Bz-Arg-OEt	H-Ala-Ser-OH	12.0	93.5
	H-Ala-Ala-Lys-Ala-Gly-OH	39.5 (51.3)	82.9
	H-Ala-Ala-Arg-Ala-Gly-OH	39.9 (52.6)	89.9
α-chymotrypsin			
Ac-Tyr-OEt	H-Gly-Leu-NH_2	69.6	97.5
	H-Ala-Ala-Phe-Ala-Gly-OH	59.9 (68.8)	89.1
	H-Ala-Ala-Tyr-Ala-Gly-OH	61.2 (71.3)	90.1
BL-GSE			
Z-Glu-OMe	H-Gly-Leu-NH_2	7.5	94.0
	H-Ala-Ala-Asp-Ala-Gly-OH	8.0	94.5
	H-Ala-Ala-Glu-Ala-Gly-OH	11.4	94.7

[a] Conditions at 25 °C: 0.1 M Hepes buffer (pH 8.0), 0.2 M NaCl, 0.02 M $CaCl_2$, [acyl donor]=2 mM, [acyl acceptor]=15 mM, [trypsin]=1.0 x 10^{-8} M, [α-chymotrypsin]=1.0 x 10^{-8} M, [BL-GSE]=1.0 x 10^{-8} M. Conditions at –15 °C: distilled water (pH 8.0 before freezing), [acyl donor]=2 mM, [acyl acceptor]=15 mM, [trypsin]=1.0 x 10^{-6} M, [α-chymotrypsin]=1.0 x 10^{-7} M, [BL-GSE]=1.0 x 10^{-8} M.

3. Conclusion

The occurrence of proteolytic side reactions during protease-mediated peptide synthesis is a compulsive result of the native activity of these enzymes for digesting of peptides and proteins. While the secondary hydrolysis of newly formed peptide bonds starts after complete acyl donor consumption and, thus, after reaching the optimum of product yield, the cleavage of specific internal bonds within the peptide reactants occurs simultaneously and competitively to the product formation. Thus, to avoid losses of intact peptide product, further efforts are essentially required leading to the minimisation of the native activity of proteases towards peptide bonds. Our results demonstrate that freezing efficiently represses the cleavage of specific peptide bonds independent

of their individual localisation and specificity achieving irreversible and efficient peptide bond formation without proteolytic side reactions. Consequently, these results confirm the distinct freezing effect on esterase and amidase activity of proteases and finally extend its appearance towards the coupling of highly specific amino acid-containing peptide fragments. Thus, from a synthetic point of view, these characteristics qualify the freezing strategy as a powerful tool in the direction of a more universal application of proteases as biocatalysts for the ligation of peptide segments. Currently, studies are in progress on the use of the freezing approach to substrate mimetic-mediated enzymatic peptide synthesis. Although, substrate mimetics considerably broaden the synthetic scope of proteases by mediating the acceptance of non-specific acyl moieties,[21–23] the use of this beneficial strategy is seriously limited to peptide reactants lacking specific amino acid residues.

4. Experimental

Materials: Amino acid derivatives, dipeptides, and synthesis chemicals were purchased from commercial suppliers. All reagents were of the highest available purity. Solvents were purified and dried by usual methods. Mass spectra of pentapeptides were recorded using thermospray ionisation with a Hewlett–Packard 5989 A instrument.

Enzymes: TLCK (N^{α}-*p*-tosyl-L-lysine chloromethyl ketone) treated bovine α-chymotrypsin (EC 3.4.21.1) and TPCK (N^{α}-*p*-tosyl-L-phenylalanine chloromethyl ketone) treated bovine trypsin (EC 3.4.21.4) were products of Fluka Chemie AG (Switzerland) and Sigma (Germany), respectively, and were used without further purification. BL-GSE was isolated from Alcalase® (Merck, Germany) according to Svendsen and Breddam with slight modifications.[24,25] The protocol involved ultrafiltration (amion hollow fibre concentrator DC2, cut off 10.000 Da) and several chromatographic purification steps using Fractogel TSK CM 650 (M) (Merck, Germany) (2.5×50 cm) and a bacitracin-sepharose 4B column (2.5×20 cm). The latter was prepared according to Stepanov and Rudenskaya.[26] The columns were equilibrated with 0.01 M Na_2HPO_4/NaH_2PO_4 buffer (pH 6.2) and 20 mM Tris buffer (pH 8.2) containing 2 mM $CaCl_2$, respectively. After washing with equilibration buffer the enzyme was eluted with linear NaCl-gradients. The two chromatographic steps were applied alternately and repeated twice. The purity of enzyme preparations were examined by SDS-PAGE and site exclusion chromatography using Superose HR 12®. BL-GSE activity was monitored spectrophotometrically at 410 nm using Ac-Glu-pNA as the substrate. Typical preparations resulted in an overall yield of 60% and had a specific BL-GSE activity of about 360 U mg^{-1} towards Ac-Glu-pNA (conditions: 0.01 M Tris–HCl buffer, pH 7.5, 5 mM $CaCl_2$, 10% dimethylformamide at 37°C). To control for undesired subtilisin impurities, the enzymatic activity of the final preparations towards Suc-Ala-Ala-Phe-pNA was checked. On the basis of these experiments, a contamination by subtilisin could be ruled out.

Chemical syntheses: Pentapeptides were synthesised with a semiautomatic batch peptide synthesiser SP 650 (Labortech AG, Switzerland) using *p*-alkoxybenzylalcohol resin, prepared according to Wang[27] and standard Fmoc chemistry. The peptides were precipitated with dry diethyl or diisopropyl ether. The purification of the final peptides was carried out by preparative HPLC or flash chromatography and recrystallisation. The identity and purity of all peptides were characterised by analytical HPLC, NMR, thermospray mass spectroscopy and elementary analysis. In all cases satisfactory analytical data (±0.4% for C, H, N) were found.

Enzymatic syntheses: Enzymatic reactions were performed in a total volume of 400 μl (25°C) and 50 μl (–15°C), respectively. Stock solutions of acyl donor esters (4 mM) were prepared in a 0.1 M Hepes buffer (pH 8.0), 0.2 M NaCl, and 20 mM $CaCl_2$ (25°C) or distilled water (–15°C). Nucleophilic pentapeptides (30 mM) were dissolved in the appropriate buffer (25°C) or distilled water (–15°C) under pH-control. If required, the pH was readjusted to 8.0 using 1N NaOH. The final concentrations of acyl donors and acyl acceptors were 2 mM and 15 mM, respectively. The latter was calculated as free, N^{α}-unprotonated nucleophilic concentration $[HN]_0$ according to the formalism of Henderson–Hasselbalch $[HN]_0 = [N]_0/(1+10^{pK-pH})$. After thermal equilibration of assay mixtures at 25°C, the reactions were initiated by addition of the appropriate enzyme stock solutions. Mixtures (50 μl) of the reactions at –15°C were precooled to 0°C and started by addition of 5 μl of the enzyme stocks. Subsequently, the mixtures were rapidly shaken and transferred into liquid nitrogen for 20 s to achieve rapid freezing. The tubes were then placed into a cryostat (Haake Q, Germany) and incubated at –15°C. Finally, the reactions were analysed by RP-HPLC. For this purpose, at defined time intervals, 50 μl aliquots were withdrawn and diluted with a quenching solution of 50% methanol containing 5% trifluoroacetic acid. Reactions at –15°C were stopped by direct addition of the quenching solution to the frozen reaction mixtures. To control for spontaneous hydrolysis and aminolysis of the acyl donor esters, parallel reactions without enzyme were analysed. On the basis of these experiments non-enzymatic aminolysis could be ruled out and the extent of spontaneous hydrolysis was found to be less than 5%. The data reported are the average of at least three independent experiments. The identity of the formed peptide products was established by thermospray mass spectroscopy.

HPLC analyses: HPLC measurements were performed using a Shimadzu LC-10A HPLC system and analysed with Shimadzu LC-10 software (Japan). A Lichrospher RP 18 column (250×4 mm, 5 μm, E. Merck, Germany) was used. Samples were eluted under isocratic or gradient conditions with various mixtures of acetonitrile and water containing 0.1% trifluoroacetic acid at flow rates of 1.0 ml min^{-1}. Detection was at 254 and 280 nm (Tyr-containing reactants), respectively, monitoring the aromatic chromophores within the acyl donors. Thus, the yields could be determined from the peak areas of acyl donor esters, hydrolysis, and aminolysis products. In the case of H-Ala-Ala-Tyr-Ala-Gly-OH, which contains with Tyr an additional chromophoric moiety, the peptide product yields were calculated from the lack of hydrolysis product using 4-nitrophenol as the internal standard.

Acknowledgements

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References

1. Schellenberger, V.; Jakubke, H.-D. *Angew. Chem.* **1991**, *103*, 1440–1452; *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 1437–1449.
2. Wong, C.-H. *Science* **1989**, *244*, 1145–1152.
3. Jackson, D. Y.; Burnier, J.; Quan, C.; Stanley, M.; Tom, J.; Wells, J. A. *Science* **1994**, *266*, 243–247.
4. Jakubke, H.-D. *J. Chin. Chem. Soc.* **1994**, *41*, 355–370.

5. Janssen, A. E. M.; Halling, P. J. *J. Am. Chem. Soc.* **1994**, *116*, 9827–9830.
6. Affleck, R.; Xu, Z.-F.; Suzawa, V.; Focht, K.; Clark, D. S.; Dordick, J. S. *Proc. Natl. Acad. Sci.* **1992**, *89*, 1100–1104.
7. Klibanov, A. M. *Trends in Biotechnol.* **1997**, *15*, 97–101.
8. Kawashiro, K.; Sugahara, H.; Sugiyama, S.; Hayashi, H. *Biotechnol. Bioeng.* **1997**, *52*, 26–31.
9. Chaudhary, A. K.; Kamat, S. V.; Beckman, E. J.; Nurok, D.; Kleyle, R. M.; Hajdu, P.; Russell, A. J. *J. Am. Chem. Soc.* **1996**, *118*, 12891–12901.
10. Grant, N. H.; Allburn, H. E. *Nature* **1966**, *212*, 194.
11. Schuster, M.; Aaviksaar, A.; Jakubke, H.-D. *Tetrahedron* **1990**, *46*, 8093–8101.
12. Schuster, M.; Ullmann, G.; Ullmann, D.; Jakubke, H.-D. *Tetrahedron Lett.* **1993**, *34*, 5701–5702.
13. Haensler, M.; Jakubke, H.-D. *Amino Acids* **1996**, *11*, 379–395.
14. Ullmann, D.; Bordusa, F.; Salchert, K.; Jakubke, H.-D. *Tetrahedron: Asymmetry* **1996**, *7*, 2047–2054.
15. Fersht, A. *Enzyme Structure & Mechanism*, 2nd ed.; W. H. Freeman & Co.: New York, 1984.
16. Fersht, A. R.; Blow, D. M.; Fastrez, J. *Biochemistry* **1973**, *12*, 2035–2041.
17. Imperiali, B.; Abeles, R. H. *Biochemistry* **1987**, *26*, 4474–4477.
18. Butenas, S.; Kalafatis, M.; Mann, K. G. *Biochemistry* **1997**, *36*, 2123–2131.
19. Haensler, M.; Jakubke, H.-D. *J. Pept. Sci.* **1996**, *2*, 279–289.
20. Pincock, R. E.; Kiovsky, T. E. *J. Chem. Educ.* **1966**, *43*, 358–360.
21. Bordusa, F.; Ullmann, D.; Elsner, C.; Jakubke, H.-D. *Angew. Chem.* **1997**, *109*, 2583–2585; *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 2473–2475.
22. Thormann, M.; Thust, S.; Hofmann, H.-J.; Bordusa, F. *Biochemistry* **1999**, *38*, 6056–6062.
23. Günther, R.; Bordusa, F. *Chem. Eur. J.* **2000**, *6*, 463–467.
24. Svendsen, I.; Breddam, K. *Eur. J. Biochem.* **1992**, *5*, 165–171.
25. Wehofsky, N.; Wissmann, J.-D.; Alisch, M.; Bordusa, F. *Biochim. Biophys. Acta* **2000**, in press.
26. Stepanov, V. M.; Rudenskaya, G. N. *J. Appl. Biochem.* **1983**, *5*, 420–428.
27. Wang, S. S. *J. Am. Chem. Soc.* **1973**, *95*, 1328–1333.