but studies designed to probe this question are in progress.

Acknowledgment. We are grateful to Dr. Charles F. Campana for his assistance with the X-ray data collection and structure determination and to the Siemens Corporation, Madison, WI, for the use of their diffractometer. The support of the National Science Foundation (Grant No. CHE 87-21657) and the U.S. Army Research Office is gratefully acknowledged.

Supplementary Material Available: Tables of crystal structure data, thermal parameters, bond distances and angles, and mean planes for **5a** (14 pages); listing of observed and calculated structure factors for **5a** (19 pages). Ordering information is given on any current masthead page.

Engineering Subtilisin for Reaction in Dimethylformamide[1]

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Received September 17, 1990

Enzyme catalysis in organic solvents has emerged as a useful technology in organic synthesis.[2] Nevertheless, problems are often encountered in such environments, particularly in polar organic solvents,[3] mainly due to the intrinsic instability and low catalytic activity of enzymes. We have recently reported the use of site-directed mutagenesis to prepare a mutant subtilisin BPN′ (subtilisin 8350) to improve the enzyme stability and activity in *N*,*N*-dimethylformamide (DMF).[4] We report here another subtilisin variant (8397) which is even more stable than the 8350 variant in DMF.

The 8350 variant contains the following amino acid substitutions which improve stabilizing interactions:[5] Asn 218 Ser (hydrogen bonding), Gly 169 Ala (hydrophobic interaction and conformational restriction), Met 50 Phe (hydrophobic interaction), Tyr 217 Lys (hydrogen bonding), Gln 206 Cys (oxidized to Cys-SH during posttranslational modification, van der Waals interaction) and Asn 76 Asp (Ca^{2+} binding and hydrogen bonding). Each of these mutations was found to have only small and localized effects on the protein structure and also to have an additive effect on the enzyme stability in aqueous media.[5] This variant was about 100 times more stable in aqueous solution and 50 times more stable in DMF than the wild-type enzyme. A marked improvement of stability in DMF was found when the Lys-217 residue of 8350 was changed back to the wild-type Tyr (Figure 1). This new variant 8397 has a half-life of 350 h in DMF and 1600 h in aqueous solution at pH 8.4 and 25 °C, compared to 20 min and 15 h, respectively, for the wild-type enzyme.[4] Since the residue at position 217 is located on the surface, change of the charged Lys back to Tyr may make the enzyme more compatible with

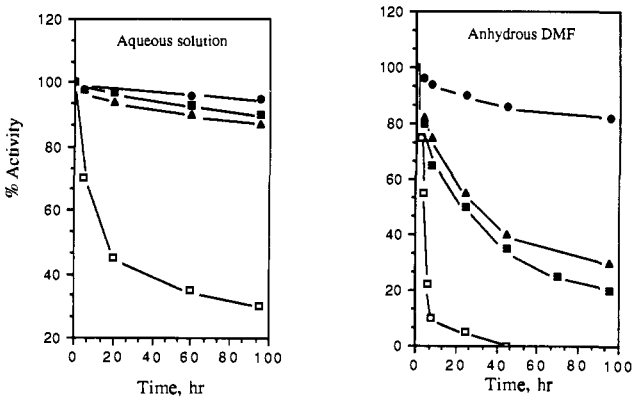

Figure 1. Stability of subtilisin BPN′ and mutants in aqueous solution (0.05 M Tris·HCl, pH 8.4) and in DMF at 25 °C: □, wild-type BPN′; ■, 8350; ●, 8397; ▲, 8399. The rate of inactivation was measured on the basis of the remaining activity as described previously.[4] The inactivation is not simple first order. 8350: Met 50 Phe (hydrophobic), Gly 169 Ala (hydrophobic and configurational entropy), Asn 76 Asp (Ca^{2+} binding and H bonding), Gln 206 Cys (oxidized to Cys-SH, van der Waals), Tyr 217 Lys (H bonding), Asn 218 Ser (H bonding). 8397: The same as 8350 except no change for Tyr 217. 8399: The same as 8350 except no changes for Gly 169 and Tyr 217.

DMF, which is a poor solvator, thereby resulting in a large, positive effect on the enzyme stability in the organic solvent. Further change of variant 8397 at position 169 from Ala to the wild-type Gly generated the mutant 8399, which is more stable than the wild-type enzyme but less stable than 8397. It had a half-life of 1000 h in aqueous solution and 43 h in DMF. The 8-fold increase in stability in DMF compared to the 3-fold increase in stability in aqueous solution for the Gly 169 Ala mutation may indicate that the increase in the conformational restriction of the enzyme in DMF (due to the loss of configurational entropy for the unfolded form)[6] seems more significant than the negative hydrophobic effect experienced in DMF vs H_2O. It is noted that the solvent-accessible surface of Gly 169 in aqueous media is only 1% as measured by water probe calculation.[7]

To examine the effect of mutations on catalysis, the kinetic parameters of 8397 and 8399 for the hydrolysis of selected esters, thioester, and amides were determined and compared to those for the wild-type enzyme and 8350 (Table I).[4] It was found that, in general, the mutations have little effect on catalysis, as indicated by the k_{cat}/K_m values. We then investigated the active-site geometry of the mutant enzymes by studying the inhibition kinetics with Boc-Ala-Val-Phe-CF_3, a designed inhibitor that forms an enzyme–inhibitor complex resembling the transition-state complex of the enzyme reaction.[4] As expected, the smaller K_i values[8] for the mutant enzymes interacting with the transition-state-based inhibitor indicate that the mutant enzymes bind to the reaction transition state of the peptide substrate slightly more strongly than the wild-type enzyme, as reflected by the higher k_{cat}/K_m values for the mutant enzymes. In addition to the L-isomer specificity at the P_1 site, we also examined the P_2 specificity of the mutant and the wild-type enzymes in the hydrolysis of a number of N-protected dipeptide esters containing a D- and an L-amino acid residue at the P_2 position.[9] All four enzymes showed a very high

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(8) Slow binding behavior as described previously[4] was observed in each case. The observed rate constant (k_{obsd}) of inhibition was thus determined and plotted vs the inhibitor concentration for K_i ($=k_{off}/k_{on}$) determination. Both k_{on} (=slope × {1 + [S]/K_m}, where [S]/K_m = 0.5 for 8399, 0.4 for 8397, and 0.45 for other enzymes), and k_{off} (intercept) were determined to be the following: wild type, $k_{on} = (1.17 \pm 0.11) \times 10^3$ M^{-1} s^{-1}, $k_{off} = (5.86 \pm 0.91) \times 10^{-3}$ s^{-1}, $K_i = 5.0$ μM; 8350, $k_{on} = (6.48 \pm 0.25) \times 10^2$ M^{-1} s^{-1}, $k_{off} = (7.17 \pm 0.22) \times 10^{-4}$ s^{-1}, $K_i = 1.05$ μM; 8397, $k_{on} = (1.26 \pm 0.03) \times 10^3$ M^{-1} s^{-1}, $k_{off} = (1.32 \pm 0.36) \times 10^{-3}$ s^{-1}, $K_i = 1.1$ μM; 8399, $k_{on} = (6.50 \pm 0.01) \times 10^2$ M^{-1} s^{-1}, $k_{off} = (4.67 \pm 0.06) \times 10^{-4}$ s^{-1}, $K_i = 7.02$ μM.
(9) The peptide esters tested were Boc-L(D)-Ala-Phe-OMe, Boc-L(D)-Phe-Phe-OMe, Boc-L(D)-Ala-Gly-OMe, and Boc-L(D)-Tyr-Gly-OMe.

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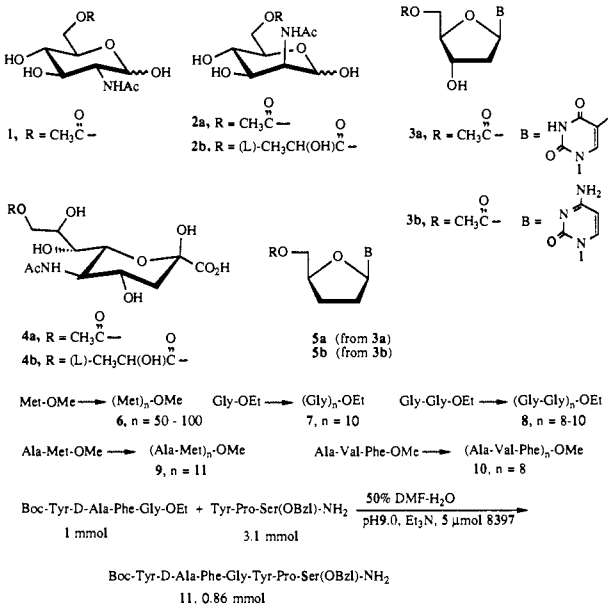
Table I. Kinetic Constants for Subtilisin BPN′ and Mutants[a]

substrate	BPN′ k_{cat}, s^{-1}	BPN′ K_m, μM	BPN′ k_{cat}/K_m, $M^{-1}\ s^{-1}$	8350 k_{cat}, s^{-1}	8350 K_m, μM	8350 k_{cat}/K_m, $M^{-1}\ s^{-1}$	8397 k_{cat}, s^{-1}	8397 K_m, μM	8397 k_{cat}/K_m, $M^{-1}\ s^{-1}$	8399 k_{cat}, s^{-1}	8399 K_m, μM	8399 k_{cat}/K_m, $M^{-1}\ s^{-1}$
Suc-AAPF-pNA[b]	47	172	2.7×10^5	130	160	8.1×10^5	74	97	7.6×10^5	76	112	6.8×10^5
NTCl[c]	0.2	76	2.2×10^3	0.6	67	9.6×10^3	0.3	42	7.1×10^3	0.2	33	6.0×10^3
Z-Lys-SBzl	46	531	8.7×10^4	33	536	6.1×10^4	70	900	7.8×10^4	32	948	3.4×10^4
Bz-Tyr-OEt	70	1700	4.1×10^4	233	818	2.9×10^5	82	2386	3.4×10^5	73	2358	3.1×10^5

[a] Conditions are the same as those described previously.[4] [b] *N*-Succinyl-Ala-Ala-Pro-Phe-*p*-nitrophenylanilide. [c] *N-trans*-Cinnamoylimidazole.

specificity for the L-amino acid at the P_2 position. The relative rates for the hydrolysis of L vs D diastereomers at an ester group are approximately >100:1.

To evaluate the synthetic utility of the mutant enzyme 8397, several regioselective reactions were conducted in DMF. Compounds **1**, **2a**, **3a**, and **3b** were prepared in 90–95% yield by reaction of the corresponding free sugars or nucleosides with 10 equiv of vinyl acetate in DMF. Compound **2b** was prepared in



50% yield with >98% regioselectivity by reaction of the corresponding free sugar with ethyl L-lactate in the presence of 10% water. Compounds **2a** and **2b** were further converted to **4a** and **4b**, respectively, via reaction with pyruvate catalyzed by sialic acid aldolase. Compounds **3a** and **3b** were deoxygenated via a radical reaction to the corresponding 2,3-dideoxy nucleosides **5a** and **5b**. The enzyme was also used in the enantioselective hydrolysis of synthetic racemic amino acid esters including *N*-(ethoxycarbonyl)furylglycine and *N*-acetylhomophenylalanine methyl esters, and the results are the same as those obtained with 8350, 8399, and the wild-type enzymes. At 50% conversion in each case, both product and the unreacted substrate were recovered in >98% ee. Application of 8397 to peptide synthesis in 50% DMF, pH 9, was also conducted,[4] and similar results were obtained for the wild-type and the three mutant enzymes, except that 8397 is about 10 times more efficient and 8350 and 8399 are about 5 times more efficient than the wild-type enzyme, presumably due to the improved stability of the mutant enzyme. Polymerization of single amino acid, dipeptide, and tripeptide methyl esters to compounds **6–10** and segment condensation for the synthesis of **11** were conducted under the same conditions.[10]

(10) The condensation product was purified and characterized to be identical with that prepared previously.[4] The polymers were purified by gel filtration chromatography, and the degree of polymerization was estimated on the basis of the molecular weight and the relative intensity of the C-terminal-OCH_3 compared to the integrated α-H's as determined by ^1H NMR. (Bibbs, J. A.; Zhong, Z.; Wong, C.-H. In *Materials Synthesis Utilizing Biological Processes*; Ricke, P. C., Calvert, P. D., Alper, M., Eds.; Materials Research Society: Pittsburgh, PA, 1990; p 223.) Short polypeptides were obtained mainly due to their low solubilities in the solvent system.

In summary, the technique of site-directed mutagenesis has proven useful for the improvement of enzyme stability in polar organic solvent. The dramatic increase in the stability of subtilisin 8397 in DMF makes it a useful enzyme for the transformation of various organic compounds which require DMF as solvent. Further study along this line should provide rich experimental data useful for engineering enzymes to be used in organic media.[11]

Supplementary Material Available: Procedures for syntheses and experimental data for **1–10** (7 pages). Ordering information is given on any current masthead page.

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Epoxyquinones from 2,5-Dihydroxyacetanilide: Opposite Facial Specificity in the Epoxidation by Enzymes from *Streptomyces* LL-C10037 and *Streptomyces* MPP 3051

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Received September 24, 1990

We have previously reported the detailed biosynthesis[1,2] of the antitumor metabolite LL-C10037α (**1**),[3] produced by *Streptomyces* LL-C10037,[4] from the shikimate pathway via 3-hydroxyanthranilic acid (**2**). Six steps from **2** to **1** were identified by whole-cell and cell-free studies,[2] with the latter implicating the acetamidoquinone **3** as the epoxidation substrate to yield epoxyquinone **4**.[5] Antibiotic MM14201 (**5**),[6] produced by *Streptomyces* MPP 3051, is the desacetyl enantiomer of **1**[3] and could be derived either by a pathway totally different from the biosynthesis of **1** or, more simply, by the same pathway (exclusive of stereochemistry) with the addition of a deacetylation as the last step. We now report that the correct epoxidase substrate is the acetamidohydroquinone **6** and that partially purified extracts from the two organisms epoxidize **6** to yield the enantiomeric products **4** and **7**, respectively.

Initially a mixture of **3** and either NADH or NADPH was treated with a cell-free extract of *S.* LL-C10037; the choice of substrate and cofactor was based on the in vivo incorporation of **3**[2] and the reported enzymatic epoxidation of nanaomycin A.[7]

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