Variants of Subtilisin BPN′ with Altered Specificity Profiles†

Michael Rheinnecker,‡ Jörg Eder,§ Pramod S. Pandey,‖ and Alan R. Fersht*

MRC Unit for Protein Function and Design, Cambridge Centre for Protein Engineering, University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, U.K.

Received August 5, 1993®

ABSTRACT: A strategy for increasing the size of the S_4 binding pocket was used to improve the specificity of subtilisin BPN′ toward substrates with large hydrophobic P_4 side chains. This approach involves single and double amino acid replacements at positions 104, 107, and 126. Previously, alteration of I107 to glycine has been found to increase the specificity of subtilisin toward leucine, isoleucine, and phenylalanine as P_4 residues by up to 214-fold. Replacement of Y104 by alanine also yields a similar improvement in specificity. However, this subtilisin variant favors isoleucine and phenylalanine over leucine. When L126 was replaced by valine, alanine, and glycine, respectively, only the L126A subtilisin variant, which possesses a 28-fold-increased catalytic efficiency for isoleucine compared with all other substrates tested, showed a significantly improved specificity profile. As inferred from the double-mutant enzymes I107G/L126V, I107G/L126A, and I107G/Y104A, none of the effects of the single amino acid replacements on the kinetic parameters are additive. The I107G/L126V mutant subtilisin has the largest improvement in P_4 substrate specificity reported so far: k_{cat}/K_M is increased 340-fold for leucine compared to alanine. By contrast, the specificity profile of the I107G/Y104A mutant enzyme is impaired in comparison with that of the corresponding single mutants. Therefore, the design of high-specificity subtilisin variants through the combination of single amino acid replacements in the S_4 pocket appears to be nontrivial due to the interference of the introduced structural changes.

Subtilisin BPN′ is a serine-class endoprotease (Markland & Smith, 1971) which is secreted by *Bacillus amyloliquefaciens*. The enzyme binds substrates with a broad specificity, mainly by interactions between its two specificity pockets, S_1[1] and S_4, and the corresponding amino acid side chains, P_1 and P_4, of the substrate. One goal over the last few years has been to engineer subtilisin BPN′ into a site-specific protease. Alteration of the electrostatic, hydrophobic, and steric properties of the S_1 pocket (Estell et al., 1986; Wells et al., 1987) and a mechanism of "substrate-assisted catalysis" (Carter & Wells, 1987; Carter et al., 1991) have successfully been used to increase the specificity toward P_1 and P_2 substrate residues, respectively. Further, the S_4 pocket is as important as the S_1 pocket in determining the substrate specificity of subtilisin BPN′, and amino acid replacements in the S_4 pocket have led to protein variants with altered specificity toward P_4 substrate residues (Bech et al., 1993; Grøn et al., 1992; Teplyakov et al., 1992; van der Laan et al., 1992; Rheinnecker et al., 1993).

So far, one of the largest improvements in substrate specificity, which is not at the expense of enzymatic activity, has been achieved by replacing I107 at the bottom of the S_4 pocket by glycine (Rheinnecker et al., 1993). Preliminary results from X-ray structure analysis of the I107G mutant subtilisin now reveal that L126, which lines the side wall of the S_4 pocket, moves toward the unoccupied space left by the I107→G mutation (Y. Harpaz, M. Rheinnecker, and A. R. Fersht, unpublished results). This movement causes alterations in the main-chain conformations of residues 125–127, which are located between the S_1 and the S_4 pocket, and also provide hydrogen bonds to the substrate. In this work, we describe the analysis of a series of amino acid replacements at position 126 in subtilisin BPN′ and the replacement of Y104 at the entrance of the S_4 pocket by alanine. A similar mutation (V104→A) has previously been shown to increase the P_4 substrate preference of subtilisin 309 toward phenylalanine (Bech et al., 1992). Analysis of the double-mutant enzymes I107G/L126V, I107G/L126A, and I107G/Y104A indicates that the changes in enzyme specificity obtained for the corresponding single amino acid replacements are nonadditive when combined.

MATERIALS AND METHODS

Materials. Restriction enzymes and other DNA-modifying enzymes were purchased from New England Biolabs. Oligonucleotides were synthesized on an Applied Biosystems ABI 380B oligonucleotide synthesizer. The substrate series s-XAPFpNA, where X represents valine, isoleucine, leucine, and phenylalanine, was synthesized as described previously (Rheinnecker et al., 1993). The substrates s-AAPFpNA and ATEE were from Sigma. All other reagents were of the highest quality commercially available.

Construction and Purification of Subtilisin Variants. The vector pDS56/RBS II (Certa et al., 1986) carrying the subtilisin gene has been described previously (Eder et al., 1993).

† M.R. was supported by the MRC and Schering Agrochemicals Ltd., J.E. by the Deutsche Forschungsgemeinschaft, and P.S.P. by the British Council.

* To whom correspondence should be addressed.

‡ Present address: MorphoSys, Frankfurter Ring 193a, 8000 München 40, Germany.

§ Present address: Sandoz Research Institute, Brunner Strasse 59, A-1235 Vienna, Austria.

‖ Present address: Department of Chemistry, Indian Institute of Technology, New Delhi 110016, India.

® Abstract published in *Advance ACS Abstracts,* December 15, 1993.

[1] Abbreviations: ATEE, acetyltyrosine ethyl ester; CI2, chymotrypsin inhibitor 2; MES, 2-(*N*-morpholino)ethanesulfonic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The binding sites on the enzyme are represented as S_4, S_3, S_2, and S_1 (Schechter & Berger, 1967) while P_4 and P_1 represent the substrate amino acid side chains that interact with the enzyme's binding sites S_4 and S_1. Substrates have the form succinyl-X-Ala-Pro-Phe-*p*-nitroanilide (where X represents the P_4 amino acid) and are abbreviated to s-XAPFpNA. Mutants are designated by the single-letter code of the wild-type amino acid followed by the residue number and the amino acid replacement.

0006-2960/94/0433-221$04.50/0

In order to construct the single-mutant enzymes L126V, L126A, L126G, and Y104A and the double-mutant enzyme I107G/Y104A, site-directed mutagenesis was performed with this plasmid in an inverse PCR reaction (Clackson et al., 1991). For expression of the corresponding subtilisin variants in *Bacillus*, the *Cla*I–*Bcl*I fragments of the resulting plasmids were subcloned between the *Cla*I and *Bcl*I restriction sites of the vector pPT30 (Thomas et al., 1985). The expression plasmids of the double-mutant proteins I107G/L126V, I107G/L126A, and I107G/L126G were constructed by ligating the *Cla*I–*Pvu*I fragment of the pPT30 plasmid, which carries the I107G mutation (Rheinnecker et al., 1993), between the *Cla*I and *Pvu*I restriction sites of the respective expression plasmids of the L126 subtilisin variants. All mutants were checked by dideoxy sequencing of the entire coding region (Sanger et al., 1977).

Wild-type and mutant subtilisins were expressed in the protease-deficient *Bacillus subtilis* strain DB104 (Kawamura & Doi, 1984). Purification of the enzymes has been described previously (Rheinnecker et al., 1993). The purified proteins were stored at −70 °C after they were dipped into liquid nitrogen. Enzyme concentrations were determined by active-site titration with the inhibitor CI2 (Rheinnecker et al., 1993).

Kinetic Characterization. The enzymes were assayed with the substrates s-XAPFpNA (Delmar et al., 1979) in 1 mL of 0.1 M Tris-HCl buffer, pH 8.6, at 25 °C with substrate concentrations varying between 0.005 and 2 mM. Reactions were initiated by the addition of 10 or 100 μL of the corresponding enzyme solution (1.5–47 μM). The kinetic parameters k_{cat} and K_M were obtained from initial rate measurements of substrate hydrolysis monitored by following the absorption at 412 nm ($\Delta\epsilon_{412} = 8480\ M^{-1}\ cm^{-1}$). Hydrolysis of the short substrate ATEE (Schwert & Takenaka, 1955) was assayed in 1 mL of 0.05 M MES buffer, pH 7.0, at 25 °C with a final enzyme concentration of 1.6 μM. The ATEE concentration was 2 mM. k_{cat}/K_M values were obtained from pseudo-first-order rate measurements monitored by following the absorption at 237 nm ($\Delta\epsilon_{237} = -225\ M^{-1}\ cm^{-1}$). Standard errors in k_{cat} and K_M for all values reported were below 10%. All data were fitted by nonlinear regression analysis using the program Enzfitter (Leatherbarrow, 1987).

RESULTS

Expression and Purification of Subtilisin Variants. All subtilisin variants, which were used in kinetic studies, were expressed and purified to >95% purity as judged by SDS–PAGE. The protein yields for subtilisin mutants L126A, L126V, and L126G were 216, 122, and 48 mg of protein/L of cell culture, respectively. The Y104A subtilisin mutant was expressed at levels of 16 mg of protein/L cell culture. All double mutants gave considerably lower protein yields, which were in the range of 5–9 mg of protein/L of cell culture for the I107G/L126A and I107G/L126V mutant subtilisin, respectively.

Secreted subtilisin gives a characteristic clear halo around colonies of *B. subtilis* harboring the subtilisin expression plasmid after overnight selection on L-agar plates containing 1% skimmed milk and kanamycin (Thomas et al., 1985). Although the expression of the I107G/L126G mutant enzyme was indicated by a similar clear halo, no detectable levels of I107G/L126G subtilisin were found even after prolonged incubation (>36 h) as judged by activity measurements on the culture medium and by SDS–PAGE. Since the expression of subtilisin *in vivo* involves processing of its pro sequence (Ikemura & Inouye, 1988), low expression levels may be due to changes in specificity. It has been shown earlier that inactive subtilisin variants, which *a priori* do not have the capacity to cleave off their own pro sequence, can be processed by addition of active subtilisin (Carter & Wells, 1987). However, addition of active subtilisin to cultures of *B. subtilis* harboring the I107G/L126G expression plasmid had no effect on the expression levels of mature I107G/L126G mutant subtilisin.

Kinetic Parameters. The steady-state kinetic parameters of the mutant subtilisins with the Ala, Val, Ile, Leu, and Phe s-XAPFpNA substrates (Ala, Val, Ile, Leu, and Phe each represent the substrate residue at the P_4 position) are summarized in Table 1. The kinetic parameters of the wild-type and the I107G mutant subtilisin have been determined previously (Rheinnecker et al., 1993) and are included in Table 1 to allow comparison with the results determined in this work.

(*a*) *L126V, L126A, and L126G Mutant Subtilisins.* The values for k_{cat} of the L126V mutant enzyme with the Ala, Leu, Ile, and Phe substrates are in the same range as the k_{cat} value for the Val substrate (1.2 s^{-1}). All k_{cat} values are at least 18-fold reduced as compared to the corresponding values for wild-type subtilisin. The K_M values show no general trend but vary from 0.32 mM for the Ala substrate to 0.06 mM for the Phe substrate. Hence, the catalytic efficiencies of the L126V mutant subtilisin indicate no clear P_4 side-chain preference: the values for k_{cat}/K_M range from 6×10^3 M^{-1} s^{-1} for the Ala substrate to 1.9×10^4 M^{-1} s^{-1} for the Ile substrate.

The L126A subtilisin variant exhibits k_{cat} values similar to those observed for the L126V mutant enzyme. The highest value for k_{cat} was obtained for the Ile substrate (1.44 s^{-1}) and the lowest for the Phe substrate (0.5 s^{-1}). In contrast to the L126V mutant subtilisin, the K_M values for the L126A mutant protein differ more strongly. Whereas the K_M values for the Ala, Val, Leu, and Phe substrates vary only between 0.25 mM for the Phe substrate and 0.84 mM for the Ala substrate, the K_M value for the Ile substrate is considerably lower (0.04 mM). As a consequence, the catalytic efficiency for the Ile substrate (0.36×10^5 M^{-1} s^{-1}) is 28-fold that for the Ala, Val, and Leu substrates and 18-fold that for the Phe substrate.

Although the K_M values of the L126G mutant subtilisin are in the same range as that of the L126A mutant enzyme, for most of the substrates the values for k_{cat} are reduced further by a factor of 10. The k_{cat} values for this variant with the Leu (0.04 s^{-1}) and Ala substrates (0.11 s^{-1}) are similar to those of the Val and Ile substrates, respectively. The k_{cat} value for the Phe substrate, however, is higher (0.64 s^{-1}). The K_M values for the Ala, Ile, Leu, and Phe substrates are in the same range as the K_M values for the Val substrate (0.78 mM). The L126G mutant subtilisin possesses a 19-fold higher catalytic efficiency for the Phe substrate (0.016×10^5 M^{-1} s^{-1}) compared with the Ala, Val, and Leu substrates.

(*b*) *Y104A Mutant Subtilisin.* The values of k_{cat} for the Y104A subtilisin variant are between 2.2 s^{-1} for the Ala substrate and 16 s^{-1} for the Phe substrate. The K_M values are higher for the Ala (1.62 mM) and Val (0.91 mM) substrates, and a 15–20-fold decrease of the K_M value was observed for the Ile and Phe substrates. The catalytic efficiencies show a clear difference between the aromatic and aliphatic side chains with a 191-fold increase in the specificity of the Y104A mutant subtilisin for a Phe side chain (2.67×10^5 M^{-1} s^{-1}) as compared to an Ala side chain (0.014×10^5 M^{-1} s^{-1}) at the P_4 position. In addition, the Y104A mutant protein discriminates by factors of 2.5 and 32 against the Ile substrate (1.01×10^5 M^{-1} s^{-1}) and the Leu substrate (0.08×10^5 M^{-1} s^{-1}), respectively, in favor of the Phe substrate.

Table 1: Activities of Wild-Type and Mutant *B. amyloliquefaciens* Subtilisin BPN′ with s-XAPFpNA Substrates[a,b]

P_4 residue X	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($\times 10^5$ M^{-1} s^{-1})	rel. specificity[d]
		Wild-Type Subtilisin[c]		
Ala	35	0.15	2.33	1
Val	34	0.08	4.25	2
Ile	30	0.08	3.75	2
Leu	24	0.06	4.0	2
Phe	36	0.06	6.0	3
		I107G[c]		
Ala	0.32	0.23	0.014	1
Val	1.5	0.23	0.065	5
Ile	8.8	0.04	2.2	157
Leu	27	0.1	2.7	193
Phe	15	0.05	3.0	214
		L126V		
Ala	1.89	0.32	0.06	1
Val	1.2	0.14	0.09	1
Ile	1.48	0.08	0.19	3
Leu	1.16	0.15	0.08	1
Phe	0.76	0.06	0.13	2
		L126A		
Ala	1.1	0.84	0.013	1
Val	0.46	0.34	0.014	1
Ile	1.44	0.04	0.36	28
Leu	0.7	0.59	0.012	1
Phe	0.5	0.25	0.02	2
		L126G		
Ala	0.11	1.29	8.5×10^{-4}	1
Val	0.05	0.78	6.9×10^{-4}	1
Ile	0.13	0.58	2.2×10^{-3}	3
Leu	0.04	0.86	4.8×10^{-4}	1
Phe	0.64	0.41	0.016	19
		Y104A		
Ala	2.2	1.62	0.014	1
Val	5.2	0.91	0.06	4
Ile	7.1	0.07	1.01	78
Leu	3.3	0.41	0.08	6
Phe	16.0	0.06	2.67	191
		I107G/Y104A		
Ala	0.3	0.29	0.01	1
Val	1.0	0.28	0.04	4
Ile	3.12	0.19	0.16	16
Leu	13.9	0.10	1.39	139
Phe	7.0	0.10	0.7	70
		I107G/L126V		
Ala	0.08	0.62	1.3×10^{-3}	1
Val	0.32	0.32	0.01	8
Ile	0.36	0.05	0.07	54
Leu	0.44	0.01	0.44	338
Phe	0.48	0.02	0.24	185
		I107G/L126A		
Ala	0.02	0.63	3.1×10^{-4}	1
Val	0.11	0.5	2.2×10^{-3}	7
Ile	0.36	0.24	0.015	48
Leu	0.88	0.14	0.063	203
Phe	0.3	0.08	0.038	123

[a] Substrate residues s-XAPFpNA correspond to succinyl-X-Ala-Pro-Phe-pNA, where X represents the P_4 substrate residues Ala, Val, Ile, Leu, or Phe. [b] Assays were performed at 25 °C in 0.1 M Tris-HCl, pH 8.6 (see Materials and Methods). [c] Data taken from Rheinnecker et al. (1993). [d] Relative specificity calculated as the ratio of the catalytic efficiencies of each substrate relative to the Ala substrate.

(*c*) *I107G/Y104A, I107G/L126V, and I107G/L126A Mutant Subtilisins.* The changes in the k_{cat} values observed upon mutation of I107 to glycine and Y104 to alanine are similar to those observed for the I107G single-mutant subtilisin. The k_{cat} values vary between 0.3 s^{-1} for the Ala substrate and 13.9 s^{-1} for the Leu substrate. Further, as seen for the I107G mutant enzyme, the K_M values decrease with increasing length of the P_4 side chain. The K_M values for the I107G/Y104A mutant subtilisin range from 0.29 mM for the Ala substrate to 0.1 mM for the Phe substrate. However, in contrast to the I107G single-mutant protein, the resulting catalytic efficiency of the I107G/Y104A mutant enzyme for the Leu substrate is 9-fold higher than that for the Ile substrate and 2-fold higher than that for the Phe substrate. The P_4 side-chain preference of the I107G/Y104A subtilisin variant is, therefore, improved compared with the I107G single-mutant subtilisin, and the enzyme discriminates more strongly against leucine, isoleucine, and phenylalanine at the P_4 position of the substrate.

Table 2: Catalytic Efficiencies of Wild-Type and Mutant Subtilisin BPN′ with Acetyl-L-tyrosine Ethyl Ester[a]

enzyme	k_{cat}/K_M ($\times 10^3$ M^{-1} s^{-1})	ATEE/s-AAPFpNA[c]
wild-type[b]	5.07	1
I107G[b]	0.35	12
L126V	2.70	20
L126A	2.70	20
L126A	0.21	7
L126G	0.14	75
Y104A	3.92	128
I107G/Y104A	0.36	67
I107G/L126V	0.21	200
I107G/L126A	0.09	18

[a] Assays were performed at 25 °C in 0.05 M MES, pH 7.0 (see Materials and Methods). [b] Data taken from Rheinnecker et al. (1993). [c] Calculated as the quotient of the percent activity with ATEE for the mutant subtilisin relative to wild-type protein and the percent activity with the s-AAPFpNA substrate for the mutant enzyme relative to wild-type subtilisin.

The k_{cat} values of the I107G/L126V mutant protein for the Val, Ile, Leu, and Phe substrates are similar (between 0.32 and 0.48 s^{-1}). However, the k_{cat} value for the Ala substrate is about 5-fold lower. The K_M values show the same general trend as observed with the I107G and I107G/Y104A mutant subtilisins and range between 0.62 and 0.01 mM for Ala and Leu as P_4 side chain, respectively. The increased catalytic efficiency toward the Leu substrate, as observed for the I107G/Y104A mutant enzyme, is even more pronounced for the I107G/L126V subtilisin variant: k_{cat}/K_M is increased by a factor of about 340 for substrates with leucine at P_4 compared to alanine at this position. The discrimination against leucine, isoleucine, and phenylalanine is in the same range as that for the I107G/Y104A mutant subtilisin.

In contrast to the I107G/L126V mutant enzyme, the k_{cat} values for the I1097G/L126A subtilisin variant follow the same trend observed for the I107G and I107G/Y104A mutant proteins. However, the k_{cat} values are considerably reduced and range from 0.02 s^{-1} for the Ala substrate to 0.88 s^{-1} for the Leu substrate. Further, the values of K_M decrease with increasing length of the hydrophobic P_4 side chain. K_M varies between 0.63 and 0.08 mM for the Ala and Phe substrates, respectively. The catalytic efficiency of the I107G/L126A mutant protein is 233-fold increased for leucine (6.3×10^3 M^{-1} s^{-1}) as compared to alanine (27 M^{-1} s^{-1}) at the P_4 position of the substrate.

(*d*) *Kinetic Parameters with ATEE.* The substrate ATEE (Schwert & Takenaka, 1955; Philipp & Bender, 1983) binds in the S_1 pocket only, and has previously been used to measure any effect of amino acid replacements in the S_4 pocket on the geometry of the catalytic triad and/or the S_1 pocket (Rheinnecker et al., 1993). Most of the mutant enzymes in this study have greatly reduced catalytic efficiencies for ATEE hydrolysis compared to wild-type subtilisin (k_{cat}/K_M = 5.07 $\times 10^3$ M^{-1} s^{-1}; Table 2). Except for the Y104A and L126V mutant enzymes, which have k_{cat}/K_M values of 3.92×10^3 and 2.7×10^3 M^{-1} s^{-1}, respectively, all other subtilisin variants

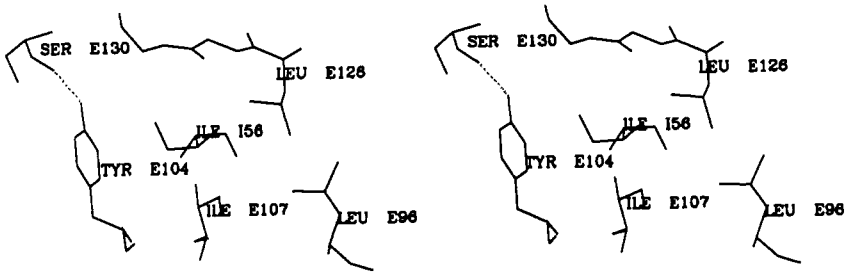

FIGURE 1: Stereographic representation of the S_4 specificity pocket of subtilisin BPN', which accommodates the P_4 residue I56 of chymotrypsin inhibitor 2. The S_4 pocket residues L96, Y104, I107, L126, and S130 are labeled.

hydrolyze the ATEE substrate with k_{cat}/K_M values in the range between 0.09×10^3 M^{-1} s^{-1} (for the I107G/L126A mutant enzyme) and 0.36×10^3 M^{-1} s^{-1} (for the I107G/Y104A mutant enzyme).

DISCUSSION

The binding of the substrates to subtilisin BPN' is influenced mainly by the interaction between the amino acid side chains at positions P_1 and P_4 of the substrate with the corresponding specificity pockets S_1 and S_4 of the enzyme (Morihara, 1974; Grøn et al., 1992; Grøn & Breddam, 1992). We have reported earlier that the replacement of I107 by glycine leads to a subtilisin BPN' variant with a larger S_4 pocket, which exhibits high specificity toward large hydrophobic side chains at the P_4 position of the substrate (Rheinnecker et al., 1993). In this study, we describe the effect of further single and double amino acid replacements in the S_4 pocket on the specificity of subtilisin. We have found that some of the mutant enzymes possess specificity improvements toward large hydrophobic P_4 side chains in the same range as the I107G subtilisin variant. In addition, these variants discriminate more strongly against isoleucine, leucine, and phenylalanine side chains in favor of leucine side chains at the P_4 position of the substrate.

The S_4 and S_1 pockets are separated by the protein backbone segment 125–127. In the crystal structure of the CI2–subtilisin complex (McPhalen et al., 1985; McPhalen & James, 1988), the majority of close-range intermolecular contacts (<4 Å) to I56, the P_4 residue of CI2, are made by I107 and Y104 (Figure 1). The removal of the hydrogen bond between Y104 and S130, brought about by changing Y104 to phenylalanine, has no significant effect on the specificity toward large P_4 substrates (Rheinnecker et al., 1993). Recently, however, mutational analysis of the equivalent position in subtilisin 309 (V104; Bech et al., 1992) revealed that an alanine residue at this position increases the specificity for phenylalanine as the P_4 side chain by a factor of 3 as compared to wild-type enzyme. Introducing a similar mutation in subtilisin BPN' (Y104A) gives this enzyme a substantially improved specificity profile. In fact, Y104A subtilisin is the only single mutant which is also as active and specific as the I107G mutant enzyme for large hydrophobic P_4 side chains. Further, this subtilisin variant shows a strong preference for phenylalanine and isoleucine over leucine side chains.

The only other amino acid side chain which makes contacts of <4 Å to the P_4 residue of CI2 is L126. The distances between the C_δ atoms of this leucine side chain and the $C_{\gamma(2)}$ atom of I56 are 3.5 and 4.2 Å. In addition, as inferred from the crystal structure of the I107G mutant subtilisin (Y. Harpaz, M. Rheinnecker, and A. R. Fersht, unpublished results), the L126 side chain moves into the cavity of the larger S_4 pocket and causes a shift in the protein backbone segment 125–127. This led us to predict that decreasing the size of the L126 side chain might increase the specificity of subtilisin BPN' toward large hydrophobic P_4 side chains through enlargement of the S_4 pocket. However, only the L126A subtilisin variant has a significantly improved specificity profile (cf. Table 1). In contrast to the I107G and Y104A mutant enzymes, this variant exhibits almost exclusive specificity toward isoleucine at the P_4 position. The catalytic efficiency is reduced by a factor of 10 compared to wild-type subtilisin. Thus, the increased specificity of this mutant enzyme is achieved with only a moderate compromise in activity.

We were also interested as to whether the combination of amino acid replacements at positions 104, 107, and 126 would further improve the specificity profile of subtilisin BPN'. All three double-mutant enzymes show strong preference for large hydrophobic P_4 side chains. However, only the I107G/L126V mutation improves the specificity of the I107G subtilisin variant further (340-fold compared to 214-fold). Moreover, all double-mutant subtilisins favor leucine at the P_4 position over isoleucine and phenylalanine. The data presented on the double-mutant enzymes indicate that none of the effects of the single amino acid replacements on the kinetic parameters are additive: the increase in specificity of the I107G/L126V, I107G/L126A, and I107G/Y104A mutant enzymes is not as high as anticipated from the observed changes in specificity on the single-position mutants. Therefore, the alteration of side chains in the S_4 pocket induces structural changes which are not independent of each other. This nonadditivity is most pronounced for the I107G/Y104A subtilisin variant where the specificity of the double-mutant enzyme is decreased compared with either of the two single mutants. In this case, the size of the S_4 pocket is probably too big to bind even large hydrophobic side chains efficiently. It was attempted to analyze this nonadditivity by means of thermodynamic cycles (Carter et al., 1984). However, a simple physical explanation was not obvious from this analysis.

The ratio of the relative activities for the hydrolysis of ATEE and s-AAPFpNA of mutants compared with wild-type subtilisin (Table 2) serves as a measure of the effect of the S_4 mutations on the S_1 pocket; the greater the ratio, the more pronounced is the effect on S_4 relative to S_1. As can be seen in Table 2, there is a large difference in this ratio: e.g., the I107G/L126V subtilisin variant has a ratio of 200 compared to only 12 for the I107G mutant subtilisin. Consequently, the effect of the S_4 mutation of the latter subtilisin variant on the S_1 pocket is much stronger than the respective effect caused by the I107G/L126V mutation.

Conclusion. The specificity of subtilisin BPN' for large hydrophobic side chains at the P_4 position of the substrate can be substantially increased by altering residues Y104, I107, and L126, which are located in the S_4 pocket. The most pronounced effects were observed for Y104A, I107G, and L126A mutant subtilisins. Further, the combination of these single amino acid replacements led to the highest increase in

specificity for one particular type of P_4 side chain reported so far. The effects of the single amino acid replacements, however, were found to be nonadditive. Therefore, any protein engineering strategy to change the specificity profile of subtilisin based on results from single-mutant studies on S_4 pocket residues may be problematical.

ACKNOWLEDGMENT

We thank Drs. A. G. Day and S. Jackson for helpful advice and discussion. We are also grateful to Drs. G. Baker and F. Saites for synthesizing the substrates and oligonucleotides, respectively.

REFERENCES

Bech, L. M., Sørensen, S. B., & Breddam, K. (1992) *Eur. J. Biochem. 209*, 869–874.
Bech, L. M., Sørensen, S. B., & Breddam, K. (1993) *Biochemistry 32*, 2845–2852.
Carter, P., & Wells, J. A. (1987) *Science 237*, 394–399.
Carter, P., Winter, G., Wilkinson, A. J., & Fersht, A. R. (1984) *Cell 38*, 835–840.
Carter, P., Abrahmsen, L., & Wells, J. A. (1991) *Biochemistry 30*, 6142–6148.
Certa, U., Bannwarth, W., Stüber, D., Gentz, R., Lanzer, M., LeGrice, S., Guillot, F., Wendler, I., Hunsmann, G., Bujard, H., & Mous, J. (1986) *EMBO J. 5*, 3051–3056.
Clackson, T., Güssow, D., & Jones, P. T. (1991) in *PCR: a practical approach* (McPherson, M. J., Quirke, P., & Taylor, G. R., Eds.) pp 187–214, IRL Press, Oxford, U.K.
Delmar, E. G., Largman, C., Brodrick, J. W., & Geokas, M. C. (1979) *Anal. Biochem. 99*, 316–320.
Eder, J., Rheinnecker, M., & Fersht, A. R. (1993) *Biochemistry 32*, 18–26.
Estell, D. A., Graycar, T. P., Miller, J. V., Powers, D. B., Burnier, J. P., Ng, P. G., & Wells, J. A. (1986) *Science 233*, 659–663.
Grøn, H., & Breddam, K. (1992) *Biochemistry 31*, 8967–8971.
Grøn, H., Morten, M., & Breddam, K. (1992) *Biochemistry 31*, 6011–6018.
Ikemura, H., & Inouye, M. (1988) *J. Biol. Chem. 263*, 12959–12963.
Kawamura, F., & Doi, R. H. (1984) *J. Bacteriol. 160*, 442–444.
Leatherbarrow, R. J. (1987) *Enzfitter*, A *Program for Nonlinear Regression Analysis*, Elsevier Scientific, New York.
Markland, F. S., & Smith, E. L. (1971) *The Enzymes* (Boyer, P. D., Ed.) Vol. III, pp 561–608, Academic Press, New York.
McPhalen, C. A., & James, N. G. (1988) *Biochemistry 27*, 6582–6598.
McPhalen, C. A., Svendsen, I., Jonassen, I., & James, M. N. G. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7242–7246.
Morihara, K. (1974) *Adv. Enzymol. Relat. Areas Mol. Biol. 41*, 179–243.
Philipp, M., & Bender, M. L. (1983) *Mol. Cell. Biochem. 51*, 5–32.
Rheinnecker, M., Baker, G., Eder, J., & Fersht, A. R. (1993) *Biochemistry 32*, 1199–1203.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.
Schechter, I., & Berger, A. (1967) *Biochem. Biophys. Res. Commun. 27*, 157–162.
Schwert, G. W., & Takenaka, Y. (1955) *Biochim. Biophys. Acta 16*, 570–575.
Teplyakov, A. V., van der Laan, J. M., Lammers, A. A., Kelders, H., Kalk, K. H., Misset, O., Mulleners, L. J. S. M., & Dijkstra, B. W. (1992) *Protein Eng. 5*, 413–420.
Thomas, P. G., Russell, A. J., & Fersht, A. R. (1985) *Nature 318*, 375–376.
Van der Laan, J. M., Teplyakov, A. V., Kelders, H., Kalk, K. H., Misset, O., Mulleners, L. J. S. M., & Dijkstra, B. W. (1992) *Protein Eng. 5*, 405–411.
Wells, J. A., Powers, D. B., Bott, R. R., Graycar, T. P., & Estell, D. A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1219–1223.