The Activity of Carboxypeptidase Y toward Substrates with Basic P_1 Amino Acid Residues Is Drastically Increased by Mutational Replacement of Leucine 178

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ABSTRACT: A random mutagenesis study on carboxypeptidase Y has previously suggested that Leu178 is situated in the S_1 binding pocket, and this has later been confirmed by the three-dimensional structure. We here report the mutational replacement of Leu178 with Trp, Phe, Ala, Ser, Cys, Asn, Asp, or Lys and the kinetic characterization of each mutant, using substrates systematically varied at the P_1 position. The general effect of these substitutions is a reduced k_{cat}/K_m for substrates with uncharged amino acid residues in the P_1 position, little effect on those with acidic residues, and an increased k_{cat}/K_m for those with basic amino acid residues. There is a clear correlation between the reduction in k_{cat}/K_m for substrates with uncharged P_1 side chains and the nature of the residue at position 178. A small reduction is observed when Leu178 is replaced by another hydrophobic amino acid residue, a larger reduction when it is replaced by a polar residue, and a very large reduction when it is replaced by a charged residue. When Leu178 is replaced by Asp, k_{cat}/K_m is reduced by a factor of 2200 for a substrate with Val in the P_1 position. The k_{cat}/K_m values for the hydrolysis of substrates with charged P_1 side chains are increased when Leu178 is replaced by an amino acid residue with the opposite charge, and they are decreased when it is replaced by a residue with the same charge. Surprisingly, all mutants (except L178K) exhibit increased activity with substrates with basic P_1 side chains. The largest increase in k_{cat}/K_m for substrates with basic P_1 side chains is observed with polar or acidic amino acid residues at position 178; e.g., with Ser and Asp k_{cat}/K_m for a substrate with Lys in the P_1 position is increased relative to the wild type by factors of 70 and 90, respectively. On the basis of all the kinetic data it is hypothesized that this increase in activity is obtained by elimination of unfavorable steric constraints present in the wild-type enzyme rather than by introduction of favorable binding contributions in the mutants. The enzyme that has Leu178 replaced by Asp exhibits the largest increase (Lys in P_1) as well as the largest decrease (Val in P_1) in k_{cat}/K_m values, and as a consequence the relative k_{cat}/K_m value has been changed by a factor of 2×10^5. It is demonstrated that the L178S and L178D enzymes, due to their altered substrate preference, can be applied in the determination of C-terminal sequences of peptides containing basic amino acids in the C-terminal region.

Carboxypeptidase Y (CPD-Y),[1] a serine carboxypeptidase of the yeast *Saccharomyces cerevisiae*, is commonly employed for digestion of peptides and proteins with the aim of determining C-terminal sequences (Breddam & Ottesen, 1987). The enzyme exhibits a wide substrate preference with respect to the P_1' position (C-terminal residue) of the substrate but a much more narrow one with respect to the P_1 position; e.g., peptides with Pro, Arg, Lys, Asp, or Glu in the penultimate position are hydrolyzed at very low rates (Breddam, 1986). This limits the applicability of the enzyme, and for this reason attempts have been made to change the P_1 substrate preference by chemical modifications within the S_1 binding pocket (Breddam, 1986). Previously, the three-dimensional structure of a homologous enzyme, carboxypeptidase II from wheat (Liao & Remington, 1990), was used to putatively assign the amino acid residues of CPD-Y involved in the formation of the S_1 binding site, and a number of mutants with increased activity toward CBZ-Lys-Leu-OH were obtained by random mutagenesis of these residues (Olesen & Kielland-Brandt, 1993). All these mutants had substitutions of residue 178, and the three-dimensional structure of CPD-Y [see Endrizzi et al. (1994)] confirms that Leu178 forms part of the S_1 binding pocket.

In order to investigate the mechanism behind the increase in activity of the position 178 mutants toward such substrates, we have constructed eight mutants by site-directed mutagenesis, in which leucine 178 has been replaced by hydrophobic amino acids of varying size (Trp, Phe, Ala), by polar amino acids (Ser, Cys, Asn) and by negatively and positively charged amino acids (Asp, Lys). All these mutant enzymes exhibit changes, some of them dramatic, in substrate preference.

MATERIALS AND METHODS

Reagents. Dipeptides, FA-*N*-hydroxysuccinimide ester, FA-Lys-Ala-OH, FA-Lys-Leu-OH, FA-Arg-Leu-OH, and FA-Phe-OMe were from Bachem AG. Oligonucleotides were synthesized on an Applied Biosystems 394 DNA/RNA

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[1] Abbreviations: Bz, *N*-benzoyl; CBZ, *N*-carbobenzoxy; CPD-WII, wheat carboxypeptidase; CPD-Y, yeast carboxypeptidase Y; dsDNA, double-stranded DNA; FA, 3-(2-furylacryloyl); kDa, kilodalton; L178X, mutant protein with leucine 178 replaced with amino acid X; ssDNA, single-stranded DNA. The binding site notation is that of Schechter and Berger (1967). Accordingly, the binding site of the C-terminal amino acid of the substrate is denoted S_1', and those of the amino acid residues in the amino-terminal direction from the scissile bond are denoted S_1, S_2, ..., S_n. In analogy, substrate residues are denoted P_1', P_1, P_2, ..., P_n.

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synthesizer. LB, 2 × YT, and SOC medium were prepared according to Sambrook et al. (1989). SC and YPD medium were prepared according to Sherman (1991).

Synthesis of FA-Xaa-Ala-OH Peptides. FA-dipeptides of the general formula FA-Xaa-Ala-OH were prepared according to the following procedure employing an active ester coupling. The dipeptide of formula H-Xaa-Ala-OH (1.05 equiv) was dissolved or usually suspended in dimethylformamide, and 1.05 equiv of triethylamine (Merck) was added. The active FA-*N*-hydroxysuccinimide ester (1.0 equiv), dissolved in dimethylformamide, was then added to give reactant concentrations around 0.3 M. The solution or slurry was then stirred at room temperature. The reaction was monitored by analytical RP-HPLC (Waters NOVOPAK 4-μm C18 column, 50 mM TEAP, pH 3.0, gradients of acetonitrile). When satisfactory conversion had been reached (between 1 and 20 h), water was added and the pH kept around 8.5 for approximately 1 h to hydrolyze the major part of unreacted FA-*N*-hydroxysuccinimide ester. The reaction mixture was desiccated and redissolved in water/ethanol. After adjustment of pH to 5.0 with HCl the reaction mixture was applied to a Waters Deltaprep with a Prepak 500 50-μm C18 column and purified by employing a gradient with increasing content of ethanol in 50 mM ammonium acetate, pH 5.0. Combined fractions containing partially purified product were desiccated, redissolved in water, adjusted to pH 3.0, and purified as above, this time by employing an increasing ethanol gradient in 50 mM acetic acid, pH 3.0. Fractions containing product of a HPLC purity of more than 98% at 220 nm were combined, desiccated, and lyophilized to produce a white amorphous powder.

Strains. Escherichia coli BMH71-18mutS (*thi supE* Δ(*lac-proAB*) [*mutS::Tn*10] F'[*proAB*$^+$ *laqI*^{q} *lacZ*ΔM15]) (Kramer et al., 1984; Zell & Fritz, 1987), *E. coli* JM109 (*recA*1 *supE*44 *endA*1 *hsdR*17 *gyrA*96 *relA*1 *thi* Δ(*lac-proAB*) F'[*traD*36 *proAB*$^+$ *laqI*^{q} *lacZ*ΔM15]) (Yanisch-Perron et al., 1985), and *S. cerevisiae* K2579LLR (Olesen & Kielland-Brandt, 1993), which is a spontaneous mutant of W2579 (*MAT***a** Δ*prc1 leu2-3 leu2-112 ura3-52 vps1-1*) (Nielsen et al., 1990) able to grow at low leucine concentrations, were used.

Transformation. Transformation of *E. coli* was performed by electroporation with a Bio-Rad Gene Pulser set at 25 μF, 200 Ω, and 2.5 kV in 2-mm cuvettes according to Dower et al. (1988). Transformation of yeast was performed according to Schiestl and Gietz (1989) and Gietz et al. (1992).

Preparation of Single-Stranded Phagemid DNA. E. coli JM109 transformed with pYSP1 (Olesen & Kielland-Brandt, 1993) was grown to an OD_{600} of 0.5 in 2 × YT + 50 mg/L tetracycline. One milliliter of this culture was superinfected with 20 μL of a $>10^9$ pfu/mL M13K07 (Dotto & Zinder, 1984) helper phage stock in a 500-mL Erlenmeyer bottle. After incubation with agitation for 1 h at 37 °C, 200 mL of 2 × YT + 50 mg/L tetracycline + 50 mg/L kanamycin were added. After incubation with agitation overnight at 37 °C, ssDNA was purified from phage particles by standard procedures (Sambrook et al., 1989). Kanamycin selects for cells superinfected with helper phage M13K07.

Mutagenesis and Sequencing. The mutagenesis was performed by a modification of the procedure by Lewis and Thompson (1990) on the plasmid pYSP1 (Olesen & Kielland-Brandt, 1993). Single-stranded pYSP1 (0.2 pmol) was mixed with 0.5 pmol of ampicillin-repair oligonucleotide (Promega, Altered Sites Kit) and 2 pmol of the mutagenic oligonucleotide in 80 μL of 1× annealing buffer (20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl), heated to 70 °C for 5 min, and allowed to cool slowly (~1 h) to room temperature. After the annealing reaction 10 μL of 10× synthesis buffer (100 mM Tris-HCl, pH 7.5, 5 mM each of the four dNTPs, 10 mM ATP, 20 mM DTT), 10 Weiss units of T4 ligase (New England Biolabs), 20 units of T4 DNA polymerase (Promega), and H_2O to 100 μL were added. The polymerase/ligation mixture was incubated at 37 °C for 2 h. Subsequently, it was phenol extracted, ethanol precipitated, resuspended, and used to transform electrocompetent *E. coli* BMH71-18mutS cells. After incubation for 45 min at 37 °C in SOC, an aliquot of the transformed cells was plated on LB with 60 mg/L ampicillin to determine the number of transformants, while the rest of the cells were grown overnight in 50 mL of LB with 60 mg/L ampicillin. A plasmid preparation from this primary culture will contain up to 50% ampicillin-sensitive plasmids originating from the unmutated parental strand. To enrich for the mutants, ~0.1 μg of miniprep DNA from this culture was used for a secondary transformation of JM109, followed by a second preparation of plasmid DNA. Sequencing was performed by the Applied Biosystems dsDNA Taq DyeDeoxy terminator procedure for use with the Applied Biosystems Model 373A DNA sequencing system to confirm the introduced mutations and to ensure that no secondary mutations had occurred.

Isolation and Kinetic Characterization of Mutant Forms of CPD-Y. Overexpression of mutant enzymes was performed according to Nielsen et al. (1990). The mutant *Bam*HI–*Bam*HI *PRC1* fragments of pYSP1 were inserted into the *GAL* expression vector pRA21 and introduced into yeast strain K2579LLR. The plasmid pRA21 is derived (R. Andersen, unpublished) from p72UG (Nielsen et al., 1990) by replacing the 918-bp *Bgl*II–*Sal*I fragment with the 638-bp *Bgl*II–*Pvu*II fragment of pWI3 (Winther et al., 1991), thereby deleting the *Bam*HI site downstream of the *PRC1* gene. Mutant forms of CPD-Y were purified from culture supernatants by affinity chromatography as described by Johansen et al. (1976). The rates of hydrolysis of FA-peptide substrates were measured spectrophotometrically at 337–352 nm (depending on substrate concentration) in 50 mM MES and 1 mM EDTA, pH 6.5, at 25 °C. Precautions were taken to avoid exposure to light due to the sensitivity of the FA group (Kanstrup & Buchardt, 1991). Kinetic parameters were determined using the GraFit 3.01 program by Erithacus Software. The influence of salt on the activity of the wild-type and L178D enzymes toward FA-Phe-Ala-OH, FA-Arg-Ala-OH, or FA-Lys-Leu-OH was measured with 0.5 mM substrate in 50 mM MES and 1 mM EDTA, pH 6.5, buffer containing various concentrations of NaCl.

Inactivation of CPD-Y by $HgCl_2$. Wild-type CPD-Y and the L178C mutant enzyme were treated by addition of 5 μL of $HgCl_2$ in water to 50 μL of enzyme (32 μM in 50 mM HEPES, pH 7.5). The final concentration of $HgCl_2$ was either 0, 9.1, 18.2, 27.3, 36.4, or 72.8 μM. The mixtures were incubated for 15 min at room temperature and then kept on ice until measurement of the residual CPD-Y activity using 0.2 mM FA-Phe-OMe in 50 mM HEPES, pH 7.5.

C-Terminal Sequencing. Digestion of 2 mM S6 phosphate receptor peptide (H-Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala-OH) (Bachem AG) was performed in 50 mM MES and 1 mM EDTA, pH 6.5, in 400-μL reactions at 25 °C with either 2.5 μM CPD-Y, 0.25 μM L178S, or 0.25 μM L178D. Samples of 40 μL were taken at selected times between 0 and 300 min, acidified by addition of 5 μL of 0.5 M HCl to terminate

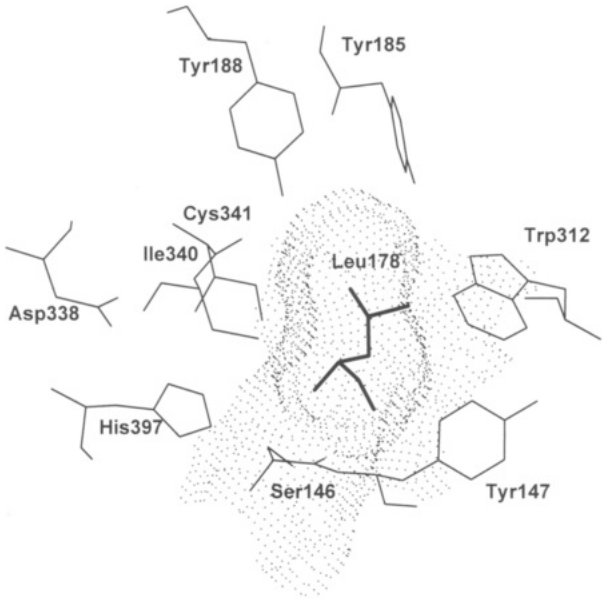

FIGURE 1: Catalytic triad (Ser146, His397, and Asp338) and S_1 binding site of CPD-Y. Leu178 constitutes the floor of the S_1 binding pocket, while Ile340 and Cys341 constitute the left side, Tyr147 and Trp312 constitute the right side, and Tyr185 and Tyr188 constitute the end. The dots indicate the solvent-accessible surface of S_1.

hydrolysis, and analyzed on a Pharmacia α-Plus amino acid analyzer.

RESULTS AND DISCUSSION

On the basis of the three-dimensional structure of the homologous enzyme carboxypeptidase W-II (Liao & Remington, 1990) and an alignment of the primary structures of the two enzymes (Breddam et al., 1987; Sørensen et al., 1987), the Leu178 residue of CPD-Y was originally hypothesized to be part of the S_1 binding pocket and hence chosen as a target for random mutagenesis (Olesen & Kielland-Brandt, 1993). The three-dimensional structure of CPD-Y is now also known [see Endrizzi et al. (1994)], and it confirms that Leu178 is in fact part of the S_1 binding pocket of CPD-Y. The structure indicates that Tyr147, Leu178, Tyr185, Tyr188, Trp312, Ile340, and Cys341 form the solvent-accessible surface of the S_1 binding pocket (Figure 1). It should be noted that when a P_1 side chain is bound to S_1, it is not totally surrounded by the binding pocket. Rather, it enters the binding pocket from one side (the direction of the catalytic Ser146) and makes contacts to the binding pocket to the left, to the right, underneath, and to the end, but it is accessible to solvent upward. Thus, if the S_1 binding pocket is viewed from the point of the catalytically essential Ser146, it can be defined as having a left side, a right side, an end, and a floor. By this definition Ile340 and Cys341 constitute the left side, Tyr147 and Trp312 the right side, Tyr185 and Tyr188 the end, and Leu178 the floor of the binding pocket (Figure 1). It is observed that the S_1 binding pocket is generally hydrophobic, which explains the preference of the enzyme for substrates with hydrophobic P_1 side chains.

To investigate the influence of residue 178 on the P_1 substrate preference, eight mutants, in which the leucine residue has been replaced by either Trp, Phe, Ala, Ser, Cys, Asn, Asp, or Lys, have been produced by site-directed mutagenesis.

In wild-type CPD-Y the distance between the γ-carbon of the side chain of Leu178 and the sulfhydryl group of Cys341 is only 4.3 Å. Thus, there is a potential for the formation of a disulfide bridge between Cys178 and Cys341 in the L178C mutant enzyme. To test this, L178C and the wild-type enzyme were treated with $HgCl_2$, which, in the absence of high anion concentration, has been shown to inhibit the wild-type enzyme by modification of the free sulfhydryl group of Cys341 (Breddam, 1983). While the wild-type enzyme is completely inhibited by one equivalent of $HgCl_2$, the L178C mutant enzyme shows no decrease in activity in the presence of more than 2 equiv of $HgCl_2$ (Figure 2), suggesting that Cys341 forms a disulfide bridge with Cys178 in L178C. The kinetic data of this enzyme, described below, indicate that the formation of this extra disulfide bridge does not cause any major structural rearrangements of this enzyme as it is kinetically very similar to L178S.

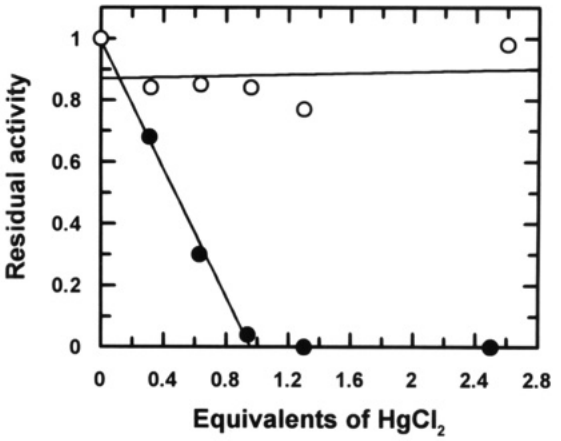

FIGURE 2: Inactivation of L178C (○) and wild-type enzyme (●) by $HgCl_2$.

The eight mutant enzymes were characterized with respect to their P_1 substrate preference using a series of FA-peptide substrates systematically varied at their P_1 position. The substrates used may be divided into two groups, one with uncharged amino acid residues (Phe, Leu, Val, Ala, or Ser) (Table 1) and the other with charged amino acid residues (Glu, Arg, or Lys) (Table 2) in the P_1 position.

All mutants exhibited reduced activity toward substrates with uncharged P_1 side chains (Table 1). The reduction is only slightly dependent on the nature of the P_1 side chain, while a clear correlation exists between k_{cat}/K_m and the nature of the amino acid residue incorporated at position 178 of the enzyme. Only a small reduction in the activity toward such substrates is observed when Leu178 is replaced by another hydrophobic residue, while a larger reduction occurs when it is replaced by a polar residue and a very large reduction is observed when it is replaced by a charged residue. As described above, the P_1 side chain is accessible to solvent upward when bound to S_1. This may explain that substituting Leu178 with another hydrophobic amino acid has only a minor effect on the activity toward substrates with hydrophobic P_1 side chains. The rotational freedom around the α–β carbon bond of the P_1 side chain secures the hydrophobic contacts with the floor of the binding pocket, even though the depth of the binding pocket has been changed according to the size of the introduced amino acid at position 178. Replacing Leu178 with a polar or charged amino acid, on the other hand, not only changes the depth of the S_1 binding pocket but also reduces its hydrophobicity, thus causing a decrease in the activity toward substrates with hydrophobic side chains.

With the other group of substrates, i.e., those with charged P_1 side chains, quite different effects were observed (Table 2). The hydrolysis of the negatively charged substrate FA-Glu-Ala-OH was only slightly influenced by replacing Leu178 with another uncharged residue (Table 2). With this

Table 1: Kinetics of CPD-Y Mutants toward Peptide Substrates with Unchanged P_1 Residues

substrate	enzyme	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (min^{-1} mM^{-1})
FA-Phe-Ala-OH	wild type	6700[a]	0.18[a]	38000[a]
	L178W	4800[a]	0.20[a]	24000[a]
	L178F	4900[a]	0.13[a]	38000[a]
	L178A	4300[a]	0.13[a]	34000[a]
	L178S	2200[a]	0.14[b]	15000[a]
	L178C	1400[a]	0.08[b]	19000[a]
	L178N	370[a]	0.13[b]	2900[b]
	L178D	1400[c]	5.2[c]	270[a]
	L178K	nd[d]	nd	420[a]
FA-Leu-Ala-OH	wild type	3800[a]	0.11[b]	35000[b]
	L178W	4600[a]	0.56[a]	8200[a]
	L178F	3600[a]	0.15[b]	23000[b]
	L178A	2100[a]	0.33[a]	6400[a]
	L178S	1300[a]	0.41[a]	3300[a]
	L178C	1000[a]	0.29[b]	3500[a]
	L178N	170[a]	0.21[a]	800[a]
	L178D	320[b]	8.4[b]	38[a]
	L178K	nd	nd	92[a]
FA-Val-Ala-OH	wild type	410[a]	0.17[a]	2400[a]
	L178W	220[a]	0.33[b]	680[a]
	L178F	110[a]	0.14[a]	770[a]
	L178A	180[a]	0.48[a]	380[a]
	L178S	30[a]	0.26[a]	120[a]
	L178C	47[a]	0.44[a]	110[a]
	L178N	9[b]	0.84[c]	11[b]
	L178D	nd	nd	1.1[a]
	L178K	nd	nd	4.1[a]
FA-Ala-Ala-OH	wild type	nd	nd	2300[a]
	L178W	590[a]	0.73[b]	810[b]
	L178F	1000[a]	1.3[a]	820[a]
	L178A	540[a]	0.65[a]	830[a]
	L178S	260[a]	0.73[b]	360[a]
	L178C	880[b]	0.84[b]	1000[a]
	L178N	67[a]	1.2[a]	56[a]
	L178D	nd	nd	6.2[a]
	L178K	nd	nd	60[a]
FA-Ser-Ala-OH	wild type	500[a]	1.0[a]	490[a]
	L178W	350[c]	2.0[c]	180[a]
	L178F	230[a]	0.84[b]	270[a]
	L178A	570[b]	1.4[c]	410[a]
	L178S	190[a]	0.93[b]	210[b]
	L178C	81[a]	0.32[b]	250[b]
	L178N	66[a]	0.88[a]	75[a]
	L178D	12[b]	1.2[b]	10[a]
	L178K	nd	nd	22[a]

[a] Standard deviation of ±0–10%. [b] Standard deviation of ±10–20%. [c] Standard deviation of ±20–30%. [d] Not determined due to high K_m.

substrate, there seems to be a correlation between the activity and the size of the introduced uncharged residue at position 178, such that mutant enzymes with large side chains exhibit less activity. Furthermore, the introduction of a positively charged residue at position 178 had a beneficial effect on the hydrolysis of this substrate while the introduction of a negatively charged residue had an adverse effect.

With substrates with basic P_1 side chains it was surprising that all mutations (except Leu178 to Lys) lead to increased activity (Table 2). A moderate increase is observed when Leu178 is replaced by another hydrophobic amino acid while a dramatic increase in activity is observed when it is replaced by either a polar or an acidic amino acid. These effects are probably not due to direct ionic interactions, since the effects of increasing the salt concentration from 0 to 0.9 M NaCl on the hydrolysis of FA-Phe-Ala-OH, FA-Arg-Ala-OH, and FA-Lys-Ala-OH with the L178D enzyme are indistinguishable from those observed with the wild-type enzyme (data not shown). Due to high K_m values with FA-Arg-Ala-OH and FA-Lys-Ala-OH k_{cat} and K_m values could not be determined

Table 2: Kinetics of CPD-Y Mutants toward Substrates with Charged P_1 Residues

substrate	enzyme	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (min^{-1} mM^{-1})
FA-Glu-Ala-OH	wild type	nd[d]	nd	41[a]
	L178W	nd	nd	3.6[a]
	L178F	nd	nd	25[a]
	L178A	nd	nd	93[a]
	L178S	nd	nd	44[a]
	L178C	nd	nd	79[a]
	L178N	nd	nd	21[a]
	L178D	nd	nd	8[a]
	L178K	nd	nd	62[a]
FA-Arg-Ala-OH	wild type	nd	nd	14[a]
	L178W	nd	nd	73[a]
	L178F	460[b]	6.3[b]	66[a]
	L178A	nd	nd	110[a]
	L1178S	5100[a]	9.2[b]	550[a]
	L178C	2100[a]	7.2[b]	300[a]
	L178N	1100[b]	7.5[c]	150[a]
	L178D	1500[a]	5.6[b]	260[a]
	L178K	nd	nd	5.8[b]
FA-Lys-Ala-OH	wild type	9[c]	5[c]	2[a]
	L178W	nd	nd	26[a]
	L178F	nd	nd	29[a]
	L178A	nd	nd	86[a]
	L178S	nd	nd	140[a]
	L178C	nd	nd	110[a]
	L178N	nd	nd	45[a]
	L178D	nd	nd	173[a]
	L178K	nd	nd	0.52[a]
FA-Arg-Leu-OH	wild type	130[a]	0.34[a]	380[a]
	L178S	6800[a]	0.78[a]	8700[a]
	L178D	1900[a]	0.45[a]	4300[a]
FA-Lys-Leu-OH	wild type	29[a]	0.68[b]	43[a]
	L178W	880[a]	2.7[b]	320[a]
	L178F	130[b]	0.76[b]	170[a]
	L178A	1100[b]	1.8[c]	590[a]
	L178S	2800[a]	1.3[b]	2100[a]
	L178C	2200[a]	2.6[b]	850[a]
	L178N	490[a]	1.2[b]	405[a]
	L178D	2300[b]	0.96[b]	2400[a]
	L178K	nd	nd	0.92[a]

[a] Standard deviation of ±0–10%. [b] Standard deviation of ±10–20%. [c] Standard deviation of ±20–30%. [d] Not determined due to high K_m.

individually. Thus, the substrates FA-Arg-Leu-OH (only with L178S and L178D) and FA-Lys-Leu-OH were used in order to investigate the nature of the increase in activity (Table 2). It is of interest to note that for all mutants the K_m values with these substrates are elevated rather than decreased, but this is more than compensated for by enormous increases in k_{cat}. Assuming that acylation is the rate-limiting step in CPD-Y-catalyzed hydrolysis of these substrates ($k_2 \ll k_3$), as is normally the case with serine protease catalyzed peptide hydrolysis (Fersht, 1985), the observed increases in k_{cat} correspond to equivalent increases in the rates of acylation. This would also imply that the wild-type enzyme actually binds such substrates better than the mutants as $K_m \simeq K_s$ under such conditions (Table 2).

The finding that the activity toward substrates with basic P_1 side chains can be increased by such varied modifications at a single position within the S_1 binding pocket is interesting and unusual. Modification of the free sulfhydryl group of Cys341 by mercuric chloride has also been shown to have a beneficial effect (in the presence of high anion concentration) on k_{cat} for the hydrolysis of such a substrate (Breddam, 1983). It is unlikely that all of these modifications could lead to similar beneficial interactions between basic P_1 residues and S_1. We hypothesize that the increase in activity has been accomplished by eliminating unfavorable steric constraints

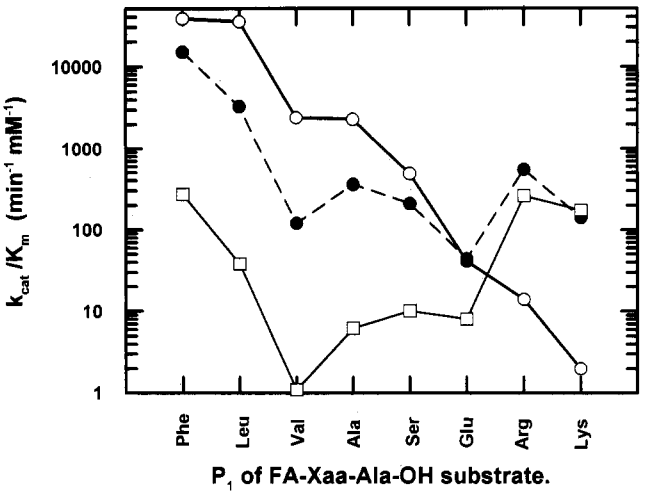

FIGURE 3: Substrate preference profiles of wild-type CPD-Y (○), L178S (●), and L178D (□). Substrates are ordered for decreasing activity with wild-type CPD-Y and connecting lines are entirely pictorial.

present in the wild-type enzyme, rather than by introducing favorable binding contributions in the mutant enzymes. This is in contrast to the few other reports in the literature where the activity of a protease toward a particular substrate has been significantly increased by site-directed mutagenesis, i.e., either by optimizing the size of a binding pocket (Bone et al., 1989; Sørensen et al., 1993) or by securing specific interactions (Hedstrom et al., 1992; Wells et al., 1987).

The most interesting enzymes of the eight mutants are L178S and L178D, which exhibit the largest changes in substrate preference (Figure 3). The k_{cat}/K_m for the hydrolysis of FA-Val-Ala-OH with the L178D enzyme has been reduced by a factor of 2200, while it has increased by a factor of 90 for FA-Lys-Ala-OH. Thus, the relative k_{cat}/K_m for these two substrates has been changed by a factor of 2×10^5, one of the largest changes in substrate preference reported for a protease. The reason that this enzyme was not identified during the random mutagenesis of position 178 (Olesen & Kielland-Brandt, 1993) is not that it is produced in extremely low amounts but rather that active CPD-Y mutants were identified on the basis of their ability to cleave a chromogenic substrate with alanine in the P_1 position, an activity which has decreased by a factor of 370 with FA-Ala-Ala-OH (Table 1).

One of many applications of CPD-Y is in determination of C-terminal peptide sequences (Breddam & Ottesen, 1987). However, the applicability is limited when the substrate preference of the enzyme does not match the C-terminal sequence to be determined, e.g., if it contains Lys or Arg residues. We have tested the L178S and L178D mutant enzymes together with the wild-type enzyme in the C-terminal sequence determination of such a peptide, the S6 phosphate receptor peptide (H-Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala-OH) (Figure 4). To the wild-type enzyme the Arg–Ala peptide bond constitutes a barrier that is hydrolyzed very slowly. As soon as Ala is released from a substrate molecule, Arg and Leu are released in quick succession. Thus, the release of Ala, Arg, and Leu is not separated in time, and hence, the sequence cannot be assigned (Figure 4, panel A). With L178S, the Arg–Ala peptide bond does not constitute a rate-limiting step, and the release of Ala, Arg, Leu, and Ser is clearly separated in time. This is due to both an increase in the rate of cleavage of the Arg–Ala bond and a decrease in that of the Leu–Arg bond. With L178D the Arg–Ala bond is cleaved much faster than the subsequent Leu–Arg bond such that Ala

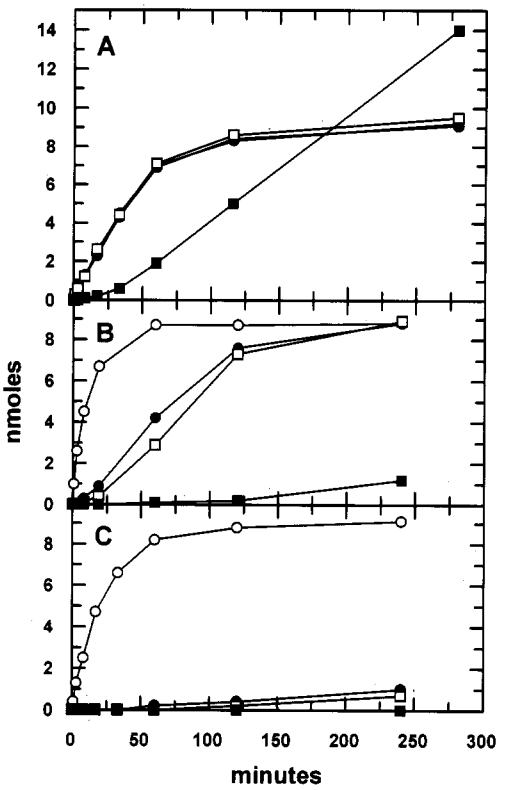

FIGURE 4: Degradation of S6 phosphate receptor peptide (H-Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala-OH) by wild-type CPD-Y (A), L178S (B), and L178D (C). Symbols: ○ = Ala, ● = Arg, □ = Leu, and ■ = Ser.

is almost fully released from the C-terminus of the peptide before the penultimate Arg follows. This represents a remarkable change in substrate preference, but for digestion of the type of peptide investigated here L178S is the preferred enzyme.

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