Substrates with Charged P_1 Residues Are Efficiently Hydrolyzed by Serine Carboxypeptidases When S_3-P_1 Interactions Are Facilitated†

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ABSTRACT: The high activity of carboxypeptidase S1 with substrates having basic P_1 residues is predicted to depend on the size of residue 312 in combination with the presence of a counter-charge in an α-helix above the S_1 binding pocket. This hypothesis is tested by the construction of 32 mutant forms of carboxypeptidase Y that combines a reduction in size of residue 312 and the introduction of either a basic or an acidic residue at either position 241 or position 245. Kinetic characterization using substrates with Leu, Arg, Lys, Glu, or Asp in P_1 demonstrates that most of these enzymes exhibit drastically altered catalytic properties. One mutant enzyme, N241D + W312L, hydrolyzes FA-Arg-Ala-OH with a k_{cat}/K_M value of 13 000 min^{-1} mM^{-1} corresponding to a 930-fold increase relative to the wild-type enzyme. This increased activity is due to an increase in k_{cat} and is independent of ionic strength. The pH profile of k_{cat}/K_M exhibits an optimum around pH 5.5 similar to that observed for CPD-S1. Another mutant enzyme, L245R + W312S, hydrolyzes FA-Glu-Ala-OH and FA-Asp-Ala-OH with k_{cat}/K_M values of 5100 and 5300 min^{-1} mM^{-1}, respectively, corresponding to 120 and 170-fold increases relative to wild-type values. With the latter substrate, a 280-fold reduction of K_M is observed. The activity of L245R + W312S is also independent of ionic strength and displays a virtually unaltered dependence on pH. The P_1 substrate preference of these two mutant enzymes for Arg versus Asp differs 2.5×10^6-fold. $\Delta\Delta G_T^{\ddagger}$ values of single and double mutants demonstrate that the effects of reducing the size of Trp312 and introducing a charged residue at position 241 or 245 in some cases exceed 100% additivity. Thus, the double mutant enzyme gains more activation energy than can be accounted for by each individual single mutation.

Serine carboxypeptidases (CPD[1]) exhibit highly varied activity toward substrates containing basic P_1 residues. With CPD-S1, from *Penicillium janthinellum*, the activity toward substrates with Lys in P_1 is 64-fold higher than with Leu (Breddam, 1988), while with CPD-Y, from *Saccharomyces cerevisiae*, it is 17 500-fold lower (Olesen *et al*., 1994). One determinant for high activity with basic P_1 residues has been shown to be residue 312 of the S_1 binding pocket (Olesen & Breddam, 1995), which in CPD-S1 is occupied by the small and hydrophilic Asn, while in CPD-Y, it is the large and hydrophobic Trp. Substitution of this residue for smaller and more hydrophilic residues, i.e., Asn, in CPD-Y increases the activity up to 1000-fold with Lys in P_1 and up to 60-fold with Arg in P_1. However, the activity levels of such mutant enzymes are still not comparable to those of CPD-S1, and thus, residue 312 is probably not the only determinant for the high activity of this enzyme with such substrates.

Analysis of the crystal structure of CPD-Y (Endrizzi *et al*., 1994) and an alignment of primary sequences of serine carboxypeptidases (Olesen & Breddam, 1995) give a hint as to the background for the high activity toward substrates with basic P_1 residues of enzymes like CPD-S1 and KEX1. Apart from having a small residue at position 312, these enzymes also possess a negatively charged residue at either position 241 or position 245. In CPD-Y, these residues reside in an α-helix just above S_1 and are probably part of S_3. However, a large residue at position 312, like in CPD-Y, prevents interaction between these residues and a P_1 side chain.

Thus, with a small residue in position 312, it may be the presence of a counter-charge at either of these positions that facilitates the binding of a charged P_1 side chain. This hypothesis was tested, both with respect to basic and acidic P_1 residues, by the construction of mutant enzymes of CPD-Y harboring mutations that reduce the size of the position 312 residue in combination with the incorporation of a counter-charge at either position 241 or position 245.

MATERIALS AND METHODS

Reagents and 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 *Saccharomyces cerevisiae* K2579LLR (Olesen & Kielland-Brandt, 1993), a spontaneous

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[1] Abbreviations: CPD, carboxypeptidase; dsDNA, double-stranded DNA; FA, 3-(2-furylacryloyl); kDa, kiloDalton; N241X, mutant protein with aspargine 241 replaced with amino acid X; L245X, mutant protein with leucine 245 replaced with amino acid X; N241X + W312Z, mutant protein with aspargine 241 replaced with amino acid X and tryptophan 312 replaced with amino acid Z; L245X + W312Z, mutant protein with leucine 245 replaced with amino acid X and tryptophan 312 replaced with amino acid Z; ssDNA, single-stranded DNA; W312X, mutant protein with tryptophan 312 replaced with amino acid X. The binding site notation is that of Schechter and Berger (1967). Accordingly, the C-terminal amino acid of the substrate is denoted P_1', and those in the amino terminal direction from the scissile bond are denoted P_1, P_2, ..., P_n. In analogy, binding sites are denoted S_1' and S_1, S_2, ..., S_n.

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mutant of W2579 (*MAT***a** Δ*prc1 leu2-3 leu2-112 ura3-52 vps1-1*) (Nielsen *et al.*, 1990) able to grow on low leucine concentrations, were from in-house stocks. LB, 2× YT, and SOC medium were prepared according to Sambrook *et al.* (1989). SC and YPD medium were prepared according to Sherman (1991). Oligonucleotides were from DNA Technology (Århus, Denmark). FA-Lys-Ala-OH, FA-Arg-Leu-OH, FA-Lys-Leu-OH, FA-Glu-Ala-OH, and FA-Asp-Ala-OH were from Bachem AG while other FA dipeptides were synthesized as previously described (Olesen *et al.*, 1994). MES was from Sigma (USA), and EDTA was from Merck (Germany). Wild-type CPD-Y was from Peptech (Hillerød, Denmark).

Transformation and Isolation of Single-Stranded DNA. 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). Single-stranded DNA of pYSP1 (Olesen & Kielland-Brandt, 1993) was prepared from a transformed culture of JM109 as described by Olesen and Kielland-Brandt (1993).

Mutagenesis and Sequencing. Mutagenesis was performed by a modification of the procedure by Lewis and Thompson (1990). 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 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 30 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 contains up to 50% ampicillin-sensitive plasmids originating from the unmutated parent strand. To enrich for mutants, 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. Mutagenic oligonucleotides were 33 bases long with the mutated codon situated at the center. Double mutants were constructed by simultaneous mutagenesis with two oligonucleotides.

Isolation of CPD-Y Mutants. Expression 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 (Olesen & Kielland-Brandt, 1993) and introduced into yeast strain K2579LLR. Mutant forms of CPD-Y were purified from culture supernatants by affinity chromatography as described by Johansen *et al.* (1976). The purity of the mutant enzymes was ascertained to be >98% by SDS−PAGE on 12.5% homogeneous gels using the PhastSystem from Pharmacia. Enzyme concentrations were calculated using $\epsilon_{280} = 88$ mM^{-1} cm^{-1} for enzymes mutated at position 312, $\epsilon_{280} = 95$ mM^{-1} cm^{-1} for all other enzymes, and MW = 64 kDa.

Kinetic Characterization. Kinetic parameters were determined in 50 mM MES and 1 mM EDTA, pH 6.5 at 25 °C. The influence of salt was performed by determination of k_{cat} and K_M values at 0 and 500 mM NaCl in 5 mM MES and 1 mM EDTA, pH 6.5 at 25 °C. The influence of pH on catalytic parameters was determined in 20 mM buffer, 1 mM EDTA, and 0.1 M NaCl at 25 °C using formic acid, acetic acid, and MES as buffers in the pH ranges 4.0−4.25, 4.25−5.5, and 5.5−6.8, respectively. Changes in buffers did not result in altered kinetic parameters with the enzymes and substrates involved. The following substrate concentrations were used: FA-Phe-Ala-OH, 0.05−0.5 mM; FA-Leu-Ala-OH and FA-Val-Ala-OH, 0.2−2.0 mM; FA-Ala-Ala-OH, 0.1−2.0 mM; FA-Ser-Ala-OH and FA-Arg-Leu-OH, 0.1−1.0 mM; FA-Glu-Ala-OH, 0.5−5.0; and 0.25−2.5 mM for the remaining substrates. Stock solutions were made by dissolving substrate in buffer and subsequently readjusting pH to 6.5. For pH profiles, a stock solution was prepared for each pH investigated. The rates of hydrolysis of FA-peptide substrates were measured spectrophotometrically at 329−352 nm, depending on substrate concentration, using a Perkin-Elmer *λ*7 spectrophotometer. Precautions were taken to avoid exposure to light due to the sensitivity of the FA group (Kanstrup & Buchardt, 1991). Kinetic parameters were determined by fitting to the Michaelis−Menten equation using the GraFit 3.09 program from Erithacus Software (Sigma). Changes in activation energy ($\Delta\Delta G_T^{\ddagger}$), the energy required for enzymatic transition of a substrate into product(s), associated with replacement of an amino acid for another, were calculated according to the formula $\Delta\Delta G_T^{\ddagger}(A\rightarrow B) = \Delta G_T^{\ddagger}(A) - \Delta G_T^{\ddagger}(B) = RT \ln(k_{cat}/K_M(A)) - RT \ln(k_{cat}/K_M(B))$ (Fersht, 1985). The pH profile of k_{cat} was fitted to the formula $k_{cat,obs} = ak_{cat,low} + (1 - a)k_{cat,high}$ where $a = 10a \log(pK_a - pH)/(1 + 10a \log(pK_a - pH))$ in case of a single pK_a while in case of two pK_a the formula $k_{cat,obs} = ak_{cat,low} + (1 - a - b)k_{cat,middle} + bk_{cat,high}$ was used where $a = cd$, $b = (1 - c)(1 - d)$, $c = 10a \log(pK_{a,1} - pH)/(10a \log(pK_{a,1} - pH) + 1)$ and $d = 10a \log(pK_{a,2} - pH)/(10a \log(pK_{a,2} - pH) + 1)$. The pH profile of K_M was fitted to the formula $K_{M,obs} = (a/K_{M,low} + (1 - a)/K_{M,high})^{-1}$ where $a = 10a \log(pK_a - pH)/(1 + 10a \log(pK_a - pH))$.

RESULTS AND DISCUSSION

Initially, a few mutant enzymes, combining N241D/K with W312A/L/F/N, were produced to determine the optimal type of residue at position 312 for accommodation of basic or acidic P_1 residues, respectively. Characterization of these enzymes indicated that for basic P_1 residues a hydrophobic residue at position 312 would be optimal while with acidic P_1 residues a hydrophilic residue would be optimal.

Altogether 39 mutant DNA forms were produced, 20 to increase the activity with basic P_1 residues by incorporating Asp or Glu at position 241 or 245 in combination with Asn, Ala, Leu, or Phe at position 312 and 19 to increase activity with acidic P_1 residues by incorporating Lys or Arg at

Table 1: Negative Charges at α-Helix above S_1

	FA-Leu-Ala-OH			FA-Arg-Ala-OH			FA-Lys-Ala-OH		
enzyme	k_{cat} (min^{-1})	K_M (mM)	k_{cat/K_M} (min^{-1} mM^{-1})	k_{cat} (min^{-1})	K_M (mM)	k_{cat/K_M} (min^{-1} mM^{-1})	k_{cat} (min^{-1})	K_M (mM)	k_{cat/K_M} (min^{-1} mM^{-1})
wild type	3800[a]	0.11[b]	35000[b]	nd[c]	nd	14[a]	nd	nd	2.0[a]
N241D	2300[a]	0.36[a]	6500[a]	nd	nd	370[a]	nd	nd	100[a]
N241D + W312A	3500[a]	0.75[a]	4700[a]	nd	nd	2100[a]	nd	nd	1500[a]
N241D + W312L	4600[a]	0.23[b]	20000[a]	nd	nd	13000[a]	nd	nd	1100[a]
N241D + W312F	3000[a]	0.37[a]	8200[a]	nd	nd	5200[a]	nd	nd	1800[a]
N241D + W312N	2500[a]	0.58[a]	4300[a]	nd	nd	2300[a]	nd	nd	920[a]
N241E	2800[a]	0.42[a]	6500[a]	nd	nd	1200[a]	nd	nd	410[a]
N241E + W312A	2800[a]	0.53[a]	5200[a]	nd	nd	260[a]	nd	nd	560[a]
N241E + W312L	5000[a]	0.35[a]	14000[a]	nd	nd	2900[a]	nd	nd	880[a]
N241E + W312F	3300[a]	0.36[a]	9200[a]	nd	nd	1400[a]	nd	nd	920[a]
L245E	2600[a]	0.27[a]	9400[a]	nd	nd	1900[a]	nd	nd	340[a]
L245E + W312A	3100[a]	0.66[a]	4700[a]	nd	nd	650[a]	3500[b]	2.3[b]	1500[a]
L245E + W312L	5700[a]	0.31[a]	18000[a]	nd	nd	3400[a]	nd	nd	800[a]
L245E + W312F	4600[a]	0.43[a]	11000[a]	nd	nd	1200[a]	nd	nd	970[a]
L245E + W312N	200[a]	1.6[b]	130[a]	nd	nd	20[a]	nd	nd	27[a]
W312A	5900[a]	0.39[b]	15000[a]	nd	nd	35[a]	nd	nd	26[a]
W312L	7200[a]	0.39[b]	18000[a]	nd	nd	410[a]	nd	nd	16[a]
W312F	5000[a]	0.26[a]	19000[a]	nd	nd	190[a]	nd	nd	8.4[a]
W312N	5000[a]	0.45[a]	11000[a]	nd	nd	53[a]	nd	nd	50[a]

[a] Standard deviation is ±0−10%. [b] Standard deviation is ±10−20%. [c] nd, not determined due to high K_M.

Table 2: Positive Charges at α-Helix above S_1

	FA-Leu-Ala-OH			FA-Glu-Ala-OH			FA-Asp-Ala-OH		
enzyme	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M (min^{-1} mM^{-1})	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M (min^{-1} mM^{-1})	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M (min^{-1} mM^{-1})
wild type	3800[a]	0.11[b]	35000[b]	nd[d]	nd	41[a]	nd	nd	31[a]
N241K	3400[a]	0.64[a]	5300[a]	nd	nd	160[a]	nd	nd	41[a]
N241K + W312A	5000[a]	0.93[a]	5300[a]	3700[b]	3.3[c]	1100[a]	140[a]	0.60[a]	230[a]
N241K + W312L	7100[a]	0.59[a]	12000[a]	810[a]	2.2[b]	360[a]	130[a]	1.1[a]	120[a]
N241K + W312F	3900[a]	0.34[a]	12000[a]	1000[a]	4.9[b]	210[a]	150[a]	1.5[b]	100[a]
N241K + W312S	4900[a]	0.98[a]	5000[a]	1800[a]	2.1[a]	890[a]	93[a]	0.70[a]	130[a]
N241K + W312N	5000[a]	0.84[a]	6000[a]	11000[a]	4.5[c]	2500[a]	130[a]	0.84[a]	160[a]
N241K + W312Q	4200[a]	0.54[b]	7800[a]	1200[a]	2.2[b]	530[a]	120[a]	1.1[b]	110[a]
N241R	4100[a]	0.67[b]	6200[a]	930[a]	3.1[a]	300[a]	110[a]	2.1[a]	55[a]
N241R + W312S	3500[a]	0.86[a]	4100[a]	3900[a]	2.1[a]	1900[a]	100[a]	0.42[b]	240[a]
N241R + W312N	3500[a]	0.90[a]	3900[a]	3300[a]	2.5[a]	1300[a]	90[a]	0.75[a]	120[a]
N241R + W312Q	3900[a]	0.52[a]	7500[a]	1900[a]	1.9[b]	1000[a]	130[a]	0.65[a]	200[a]
L245K	3000[a]	0.56[b]	5400[a]	nd	nd	190[a]	220[a]	1.8[b]	130[a]
L245K + W312N	3500[a]	1.5[a]	2300[a]	1800[a]	1.2[b]	1600[a]	140[a]	0.13[a]	1000[a]
L245K + W312Q	4000[a]	1.1[a]	3500[a]	1900[a]	1.8[a]	1100[a]	150[a]	0.12[a]	1300[a]
L245R	2900[a]	0.54[a]	5200[a]	840[a]	1.5[b]	570[a]	830[a]	0.30[a]	2800[a]
L245R + W312S	5200[a]	0.68[a]	7700[a]	1600[a]	0.32[a]	5100[a]	180[a]	0.035[a]	5300[a]
L245R + W312N	5200[a]	0.72[a]	7200[a]	2000[a]	0.44[b]	4500[a]	360[a]	0.11[a]	3300[a]
L245R + W312Q	7900[a]	0.78[a]	10000[a]	790[a]	0.48[a]	1600[a]	510[a]	0.14[a]	3700[a]
W312A	5900[a]	0.39[a]	15000[a]	nd	nd	110[a]			
W312L	7200[a]	0.39[b]	18000[a]	nd	nd	160[a]			
W312F	5000[a]	0.26[a]	19000[a]	nd	nd	120[a]			
W312S	3600[a]	0.51[a]	7000[a]	nd	nd	84[a]			
W312N	5000[a]	0.45[a]	11000[a]	nd	nd	85[a]			
W312Q	9700[a]	0.44[a]	22000[a]	nd	nd	130[a]			

[a] Standard deviation is ±0−10%. [b] Standard deviation is ±10−20%. [c] Standard deviation is ±20−30%. [d] nd, not determined due to high K_M.

position 241 or 245 in combination with Ser, Asn, Gln, Ala, Leu, or Phe at position 312. Among these L245K + W312S, N241E + W312N, and all enzymes incorporating the L245D mutation could not be expressed at detectable levels. The remaining 32 enzymes were purified by affinity chromatography and kinetically characterized using FA-Leu-Ala-OH, FA-Arg-Ala-OH, and FA-Lys-Ala-OH as substrates (Table 1) or FA-Leu-Ala-OH, FA-Glu-Ala-OH, and FA-Asp-Ala-OH as substrates (Table 2).

Kinetic Characterization. The general effect of introducing a negative charge at positions 241 or 245 is a 5-fold decrease in the activity (k_{cat}/K_M) with FA-Leu-Ala-OH as a result of increased K_M values and a 30−200-fold increase in activity with FA-Arg-Ala-OH and FA-Lys-Ala-OH (Table 1). Combining these mutations with a reduction in size of the position 312 residue has little effect on activity with Leu in P_1 but does, as expected, in most cases result in further increases in activity with basic P_1 residues. The highest

Table 3: Observed and Calculated $\Delta\Delta G_T^{\ddagger}$(Wt→mutant) Values for Mutants with Negative Charges at α-Helix above S_1 (kJ/mol)

	FA-Leu-Ala-OH			FA-Arg-Ala-OH			FA-Lys-Ala-OH		
enzyme	obs	sum	dif	obs	sum	dif	obs	sum	dif
N241D	4.2[b]	na[c]	na	−8.1[a]	na	na	−9.7[a]	na	na
N241D + W312A	5.0[b]	6.3[b]	−1.3[b]	−12.4[a]	−10.4[a]	2.0[b]	−16.4[a]	−16.1[a]	0.4[a]
N241D + W312L	1.4[b]	5.8[b]	−4.4[b]	−16.9[a]	−16.5[a]	0.5[b]	−15.6[a]	−14.9[a]	0.8[a]
N241D + W312F	3.6[b]	5.7[b]	−2.1[b]	−14.7[a]	−14.6[a]	0.1[b]	−16.9[a]	−13.3[a]	3.6[b]
N241D + W312N	5.2[b]	7.0[b]	−1.8[b]	−12.6[a]	−11.4[a]	1.2[b]	−15.2[a]	−17.7[a]	−2.5[a]
N241E	4.2[b]	na	na	−11.0[a]	na	na	−13.2[a]	na	na
N241E + W312A	4.7[b]	6.3[b]	−1.6[b]	−7.2[a]	−13.3[b]	−6.1[b]	−14.0[a]	−19.6[a]	−5.6[a]
N241E + W312L	2.3[b]	5.8[b]	−3.6[b]	−13.2[a]	−19.4[b]	−6.2[b]	−15.1[a]	−18.3[a]	−3.3[b]
N241E + W312F	3.3[b]	5.7[b]	−2.4[b]	−11.4[a]	−17.5[b]	−6.1[b]	−15.2[a]	−16.8[a]	−1.6[a]
L245E	3.3[b]	na	na	−12.2[a]	na	na	−12.7[a]	na	na
L245E + W312A	5.0[b]	5.4[b]	−0.4[b]	−9.5[a]	−14.4[a]	−4.9[b]	−16.4[a]	−19.1[a]	−2.7[b]
L245E + W312L	1.6[b]	4.9[b]	−3.3[b]	−13.6[b]	−20.5[a]	−6.9[b]	−14.9[a]	−17.9[a]	−3.0[b]
L245E + W312F	2.9[b]	4.8[b]	−1.9[b]	−11.0[a]	−18.6[a]	−7.6[b]	−15.3[a]	−16.3[a]	−1.0[b]
L245E + W312N	13.9[b]	6.1[b]	7.7[b]	−0.9[a]	−15.5[b]	−14.6[b]	−6.5[a]	−20.7[a]	−14.3[a]
W312A	2.1[b]	na	na	−2.3[a]	na	na	−6.4[a]	na	na
W312L	1.6[b]	na	na	−8.4[a]	na	na	−5.2[a]	na	na
W312F	1.5[b]	na	na	−6.5[a]	na	na	−3.6[a]	na	na
W312N	2.9[b]	na	na	−3.3[a]	na	na	−8.0[a]	na	na

[a] Standard deviation is ±0−0.1 kJ/mol. [b] Standard deviation is ±0.1−0.2 kJ/mol. [c] na, not applicable.

activity with such substrates is found with N241D + W312L and FA-Arg-Ala-OH in which case a k_{cat}/K_M value of 13 000 min^{-1} mM^{-1} is observed. This corresponds to a 930-fold increase in activity as compared to the wild-type enzyme. The increase in activity is primarily a result of increased k_{cat} values as previously observed with other mutations within the S_1 subsite that result in increased activity with such substrates (Olesen & Kielland-Brandt, 1993; Olesen *et al*., 1994; Olesen & Breddam, 1995). Although K_M with N241D + W312L and FA-Arg-Ala-OH exceeds the maximum substrate concentration, apparently k_{cat} reaches a remarkable 60 000 min^{-1}, which is 15 times that obtained with wild-type enzyme and FA-Leu-Ala-OH. To verify this high turnover number, the kinetic parameters of N241D + W312L with FA-Arg-Leu-OH was also determined. With this substrate the following values were found: k_{cat} = 69 000 min^{-1}, K_M = 0.82 mM, and k_{cat}/K_M = 85 000 min^{-1} mM^{-1}.

When positive charges were introduced at position 241 or 245, the general effect was also a ∼5-fold decrease in activity with FA-Leu-Ala-OH, again as a result of increased K_M values. The activity with acidic P_1 residues is increased 1.3−5-fold with N241K, N241R, and L245K and 90-fold with L245R. In contrast to the increased k_{cat} values observed with basic P_1 residues, the increased activity with acidic P_1 residues are in all cases primarily a result of decreased K_M values. Combining these mutations with mutations at position 312 has very little effect on activity with FA-Leu-Ala-OH whereas the activity with acidic P_1 residues in some cases increases another 10−15-fold. The highest activities with such substrates are observed with L245R + W312S in which case FA-Glu-Ala-OH and FA-Asp-Ala-OH are hydrolyzed with k_{cat}/K_M values of 5100 and 5300 min^{-1} mM^{-1}, respectively, corresponding to 120- and 170-fold increases as compared to the wild-type values. With the latter substrate, K_M has decreased 280-fold.

Additivity. The effects of combining substitutions at position 241 or 245 with substitutions at position 312 on the activity with FA-Leu-Ala-OH are minimal. When comparing the $\Delta\Delta G_T^{\ddagger}$ values (see Materials and Methods) of single and double mutants, it is observed that the $\Delta\Delta G_T^{\ddagger}$ values of double mutants are smaller than the sum of $\Delta\Delta G_T^{\ddagger}$ values of the corresponding single mutants (Tables 3 and 4). Thus, with FA-Leu-Ala-OH the effects of these mutations are not additive. Likewise, with basic P_1 residues the $\Delta\Delta G_T^{\ddagger}$ values with most double mutants are also smaller than the sum of $\Delta\Delta G_T^{\ddagger}$ values of corresponding single mutants (Table 3). However with N241D + W312L, FA-Arg-Ala-OH, and FA-Lys-Ala-OH additivity is observed. Interestingly, a few cases of "larger than additive" effects are also observed, i.e., with N241D + W312F and FA-Lys-Ala-OH the $\Delta\Delta G_T^{\ddagger}$ value of −16.9 kJ/mol with the double mutant exceeds the sum of $\Delta\Delta G_T^{\ddagger}$ values of single mutants by 3.6 kJ/mol. Such larger than additive effects are observed in most cases with acidic P_1 residues, e.g., the $\Delta\Delta G_T^{\ddagger}$ value with N241K + W312N and FA-Glu-Ala-OH of −10.2 kJ/mole exceeds the sum of $\Delta\Delta G_T^{\ddagger}$ values of single mutants by 4.6 kJ/mol (Table 4). This reflects that the beneficial effect of introducing a counter-charge at position 241 or 245 cannot be fully utilized when a bulky Trp at position 312 shields P_1 from S_3. Only when the size of the residue 312 is reduced can this S_3−P_1 interaction be maximized.

Substrate Preference. The two enzymes with the highest k_{cat}/K_M values with basic and acidic P_1 residues, N241D + W312L and L245R + W312S, respectively, were chosen for further kinetic characterization. Both enzymes were characterized for their P_1 substrate preference using FA-Xaa-Ala-OH substrates with Phe, Leu, Val, Ala, Ser, Arg, Lys, Glu, or Asp at P_1 (Table 5). Generally, with other hydrophobic residues at P_1 the activity decreases slightly as observed with Leu whereas the activity with Ser in P_1 is essentially unaffected. With N241D + W312L the activity with acidic P_1 residues is only slightly affected, while with L245R + W312S the activity with Arg in P_1 decreases 14-fold but surprisingly increases 5-fold with Lys. However, this reflects that with the W312S enzyme Lys in P_1 is also better accommodated than Arg (Olesen & Breddam, 1995). Thus, as the activity with these two enzymes with FA-Arg-Ala-OH and FA-Asp-Ala-OH differs 13 000-fold and 190-fold, respectively, their P_1 substrate preference for Arg versus Asp differs 2.5 × 10^6-fold.

Dependence on Ionic Strength. The high activities of enzymes like CPD-S1, N241D + W312L, and L245R +

Table 4: Observed and Calculated $\Delta\Delta G_T^{\ddagger}$(Wt→mutant) Values for Mutants with Positive Charges at α-Helix above S_1 (kJ/mol)

	FA-Leu-Ala-OH			FA-Glu-Ala-OH			FA-Asp-Ala-OH
enzyme	obs	sum	dif	obs	sum	dif	obs
N241K	4.7[b]	na[c]	na	−3.8[a]	na	na	−0.7[a]
N241K + W312A	4.7[b]	6.8[b]	−2.1[b]	−8.2[a]	−6.2[a]	1.9[a]	−5.0[a]
N241K + W312L	2.7[b]	6.3[b]	−3.7[b]	−5.4[a]	−7.2[a]	−1.8[a]	−3.4[a]
N241K + W312F	2.7[b]	6.2[b]	−3.5[b]	−4.0[a]	−6.5[a]	−2.4[a]	−2.9[a]
N241K + W312S	4.8[b]	8.7[b]	−3.8[b]	−7.6[a]	−5.6[a]	2.0[a]	−3.6[a]
N241K + W312N	4.4[b]	7.5[b]	−3.2[b]	−10.2[a]	−5.6[a]	4.6[a]	−4.1[a]
N241K + W312Q	3.7[b]	5.8[b]	−2.1[b]	−6.3[a]	−6.7[a]	−0.3[a]	−3.1[a]
N241R	4.3[b]	na	na	−4.9[a]	na	na	−1.4[a]
N241R + W312S	5.3[b]	8.3[b]	−3.0[b]	−9.5[a]	−6.7[a]	2.8[a]	−5.1[a]
N241R + W312N	5.4[b]	7.2[b]	−1.7[b]	−8.6[a]	−6.7[a]	1.8[a]	−3.4[a]
N241R + W312Q	3.8[b]	5.4[b]	−1.6[b]	−7.9[a]	−7.8[a]	0.1[a]	−4.6[a]
L245K	4.6[b]	na	na	−3.8[a]	na	na	−3.6[a]
L245K + W312N	6.7[b]	7.5[b]	−0.8[b]	−9.1[a]	−5.6[a]	3.5[a]	−8.6[a]
L245K + W312Q	5.7[b]	5.8[b]	−0.1[b]	−8.2[a]	−6.7[a]	1.5[a]	−9.3[a]
L245R	4.7[b]	na	na	−6.5[a]	na	na	−10.8[a]
L245R + W312S	3.8[b]	8.7[b]	−5.0[b]	−12.0[a]	−8.3[a]	3.7[a]	−12.7[a]
L245R + W312N	3.9[b]	7.6[b]	−3.7[b]	−11.6[a]	−8.3[a]	3.3[b]	−11.6[a]
L245R + W312Q	3.1[b]	5.9[b]	−2.8[b]	−9.1[a]	−9.4[a]	−0.3[a]	−11.9[a]
W312A	2.1[b]	na	na	−2.4[a]	na	na	nd
W312L	1.6[b]	na	na	−3.4[a]	na	na	nd
W312F	1.5[b]	na	na	−2.7[a]	na	na	nd
W312S	4.0[b]	na	na	−1.8[a]	na	na	nd
W312N	2.9[b]	na	na	−1.8[a]	na	na	nd
W312Q	1.2[b]	na	na	−2.9[a]	na	na	nd

[a] Standard deviation is ±0−0.1 kJ/mol. [b] Standard deviation is ±0.1−0.2 kJ/mol. [c] na, not applicable.

Table 5: Kinetics of Two Selected Mutant Enzymes

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]
	N241D + W312L	5300[a]	0.33[a]	16000[a]
	L245R + W312S	1500[a]	0.55[a]	2800[a]
FA-Leu-Ala-OH	wild type	3800[a]	0.11[b]	35000[b]
	N241D + W312L	4600[a]	0.23[b]	20000[a]
	L245R + W312S	5200[a]	0.68[a]	7700[a]
FA-Val-Ala-OH	wild type	410[a]	0.17[a]	2400[a]
	N241D + W312L	85[a]	0.33[a]	250[a]
	L245R+ W312S	280[a]	0.77[a]	360[a]
FA-Ala-Ala-OH	wild type	nd[c]	nd	2300[a]
	N241D + W312L	nd	nd	360[a]
	L245R + W312S	nd	nd	560[a]
FA-Ser-Ala-OH	wild type	500[a]	1.0[a]	490[a]
	N241D + W312L	140[a]	0.46[a]	300[a]
	L245R + W312S	nd	nd	280[a]
FA-Arg-Ala-OH	wild type	nd	nd	14[a]
	N241D + W312L	nd	nd	13000[a]
	L245R + W312S	1.7[b]	1.7[b]	1.0[b]
FA-Lys-Ala-OH	wild type	nd	nd	2.0[a]
	N241D + W312L	nd	nd	1100[a]
	L245R + W312S	nd	nd	10[a]
FA-Glu-Ala-OH	wild type	nd	nd	41[a]
	N241D + W312L	400[a]	4.4[b]	91[a]
	L245R + W312S	1600[a]	0.32[a]	5100[a]
FA-Asp-Ala-OH	wild type	nd	nd	31[a]
	N241D + W312L	72[a]	2.6[b]	28[a]
	L245R + W312S	180[a]	0.035[a]	5300[a]

[a] Standard deviation is ±0−10%. [b] Standard deviation is ±10−20%. [c] nd, not determined due to high K_m.

W312S with substrates with charged P_1 residues suggest that salt bridges might form between the involved charged residues in S_3 and P_1. However, for CPD-S1 this has been shown not to be the case (Breddam, 1988). Thus, to investigate whether the activity of the two mutant enzymes depends on such interaction, the influence of ionic strength on activity was measured using FA-Ala-Leu-OH and FA-Lys-Leu-OH or FA-Glu-Ala-OH as substrates. The catalytic parameters were determined in 5 mM MES buffer with 0 or 500 mM NaCl (Table 6). With wild-type enzyme, only small changes in catalytic parameters are observed with FA-Ala-Leu-OH and FA-Glu-Ala-OH, while with FA-Lys-Leu-OH, k_{cat} increases 3-fold and K_M increases 2-fold from 0 to 500 mM NaCl. Likewise, catalytic parameters vary little for N241D + W312L with FA-Ala-Leu-OH; with FA-Lys-Leu-OH, k_{cat}/K_M decreases 2-fold. With L245R + W312S and FA-Ala-Leu-OH, k_{cat} and K_M increases 1.6- and 1.9-fold, respectively, while with FA-Glu-Ala-OH, K_M increases 1.8-fold. None of these variations in catalytic parameters with 0 and 500 mM NaCl support the idea that salt bridges exist between S_3 and P_1 in these enzyme/substrate complexes, and hence both mutant enzymes behave like CPD-S1 in this respect.

Dependence of pH. Despite the apparent lack of ionic interactions between charged P_1 and S_3 residues, the degree of ionization of these residues should still influence catalytic parameters and hence substrate preference. The pH profiles of k_{cat} and K_M were determined for CPD-Y with FA-Ala-Leu-OH, for N241D + W312L with FA-Ala-Leu-OH and FA-Lys-Leu-OH (Figure 1), and for L245R + W312S with FA-Ala-Leu-OH and FA-Glu-Ala-OH (Figure 2) in the pH range 4−7. It was not possible to investigate the influence of protonation of the basic residues involved due to the high pK_a of such residues, and due to high K_M values with CPD-Y and charged P_1 residues, it was not possible to investigate the pH dependence of this enzyme with such substrates. Substrates were selected primarily on technical grounds, i.e., K_M should be in the range of 0.02−2 mM within the applied pH range of 4−7, thus FA-Ala-Leu-OH was chosen rather than FA-Leu-Ala-OH due to the higher K_M values obtained with this substrate, while FA-Lys-Leu-OH was chosen rather than FA-Lys-Ala-OH due to lower K_M values. The variation between Ala and Leu in the P_1 position does not affect the shape of the pH profiles, only the absolute values.

With FA-Ala-Leu-OH, all three enzymes show similar pH profiles of k_{cat} and similar pH profiles of K_M. Thus, with

Table 6: Influence of Salt on Catalytic Parameters

		0 mM NaCl			500 mM NaCl		
enzyme	substrate	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M (min^{-1} mM^{-1})	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M (min^{-1} mM^{-1})
wild type	FA-Ala-Leu-OH	42000[a]	0.52[a]	81000[a]	39000[a]	0.46[b]	86000[a]
	FA-Lys-Leu-OH	17[a]	0.34[a]	50[a]	52[a]	0.66[a]	79[a]
	FA-Glu-Ala-OH	nd[c]	nd	63[a]	nd	nd	45[a]
N241D + W312L	FA-Ala-Leu-OH	4300[a]	2.0[a]	2200[a]	4500[a]	1.5[b]	3000[a]
	FA-Lys-Leu-OH	7800[a]	0.71[a]	11000[a]	6200[a]	1.1[b]	5600[a]
L245R + W312S	FA-Ala-Leu-OH	nd	nd	4300[a]	nd	nd	4900[a]
	FA-Glu-Ala-OH	1600[a]	0.61[a]	2600[a]	1500[a]	1.1[a]	1400[a]

[a] Standard deviation is ±0−10%. [b] Standard deviation is ±10−20%. [c] nd, not determined due to high K_M.

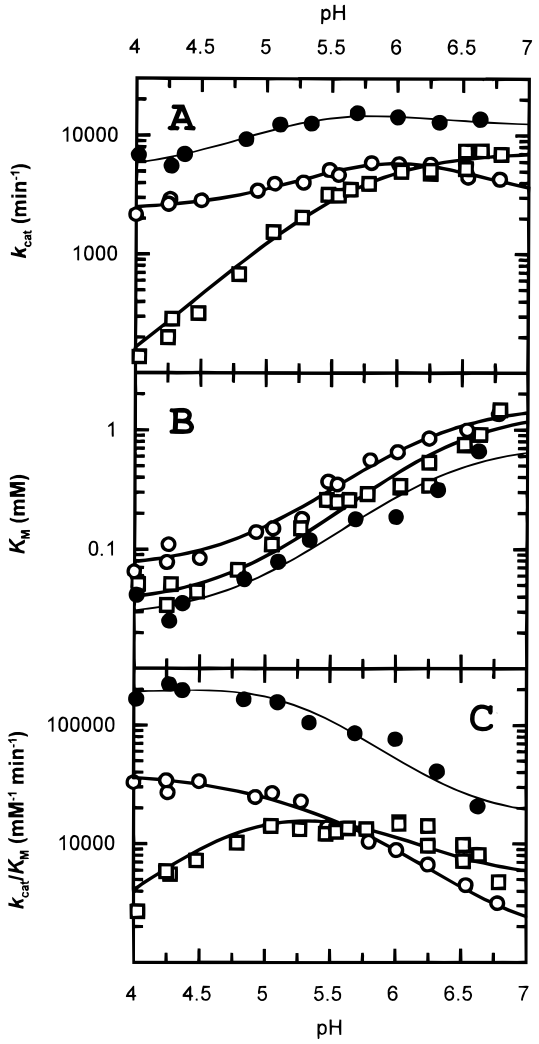

FIGURE 1: pH dependencies of k_{cat}, K_M, and k_{cat}/K_M for the hydrolysis of FA-Ala-Leu-OH (●) with CPD-Y and FA-Ala-Leu-OH (○) and FA-Lys-Leu-OH (□) with N241D + W312L. With FA-Ala-Leu-OH and CPD-Y, k_{cat} fits the deprotonation of two groups with pK_a values of 5.3 ± 0.2 and 5.8 ± 0.3, respectively, while with N241D + W312L it fits the deprotonation of two groups with pK_a values of 5.9 ± 0.5, and 6.0 ± 0.5 respectively. With FA-Lys-Leu-OH and N241D + W312L, k_{cat} fits the deprotonation of a single group with a pK_a of 5.7 ± 0.1. With all three substrate/enzyme combinations, K_M fits the deprotonation of a single group with a pK_a value of 4.8 ± 0.2.

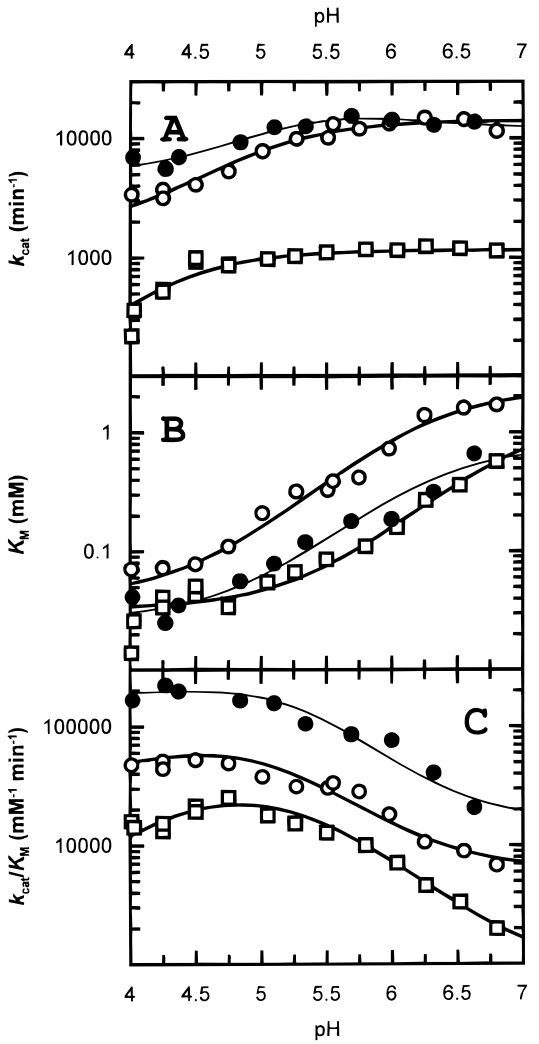

FIGURE 2: pH dependencies of k_{cat}, K_M, and k_{cat}/K_M for the hydrolysis of FA-Ala-Leu-OH (●) with CPD-Y and FA-Ala-Leu-OH (○) and FA-Glu-Ala-OH (□) with L245R + W312S. With FA-Ala-Leu-OH and CPD-Y, k_{cat} fits the deprotonation of two groups with pK_a values of 5.3 ± 0.2 and 5.8 ± 0.3, respectively, while with L245R + W312S it fits the deprotonation of a single group with a pK_a value of 5.0 ± 0.2. With FA-Glu-Ala-OH and L245R + W312S, k_{cat} fits the deprotonation of a single group with a pK_a value of 4.3 ± 0.1. With FA-Ala-Leu-OH and CPD-Y, K_M fits the deprotonation of a single group with a pK_a value of 4.8 ± 0.2, while with L245R + W312S, it fits the deprotonation of a single group with a pK_a value of 4.5 ± 0.5. With FA-Glu-Ala-OH and L245R + W312S, K_M fits the deprotonation of a single group with a pK_a value of 5.4 ± 0.1.

this substrate, no effect of the deprotonation of Asp241 can be observed.

With FA-Glu-Ala-OH and L245R + W312S, the pH profile of k_{cat} fit the deprotonation of a residue with a pK_a value about 1 pH unit lower than with FA-Ala-Leu-OH while the pH profile of K_M fit the deprotonation of a residue with a pK_a value 0.5 pH unit higher. As a consequence, optimum k_{cat}/K_M is observed at pH 4.75 rather than pH 4.0.

With FA-Lys-Leu-OH and N241D + W312L, the dependence of K_M is very similar to that observed with FA-Ala-Leu-OH. However, the dependence of k_{cat} is very different since it fits to the deprotonation of a single residue with a p$K_a = 5.7 \pm 0.1$ and with a maximum k_{cat} at high pH. The deprotonating group must be assigned to Asp241, and thus the pK_a of this side chain is perturbed about 1.8 pH units relative to the free form of aspartic acid. Also, it is evident from this pH profile that it is the actual charge of Asp241 that leads to increased k_{cat} values with basic P_1 side chains. As a consequence of this altered pH dependence, optimum k_{cat}/K_M is observed at pH 5.5 similar to previous observations with CPD-S1 using similar substrates (Breddam, 1988). Thus, the preference of this enzyme for hydrophobic versus basic P_1 residues varies with pH. At pH 6−7, it displays a 2-fold preference for Lys relative to Ala, while at pH 4.0 the preference is reversed to a 10-fold preference for Ala relative to Lys. At lower pH this preference appears to increase even further.

Concluding Remarks. The activity of serine carboxypeptidases (sharing the CPD-Y like fold) with substrates having charged P_1 residues has been shown to depend on the nature of residues 241, 245, and 312. With basic P_1 residues the highest activity is obtained when residue 312 is small and either residue 241 or residue 245 is acidic, as it is the case for enzymes like CPD-S1 and KEX1. Furthermore, such a mutant form of CPD-Y displays the same high activity, independence of salt, and dependence of pH as CPD-S1. Consequently, it can be predicted that the putative enzymes YBY9 of yeast *Saccharomyces cerevisiae* (Becham *et al*., 1994), CPD-Z of the zygomycete *Absidia zychae* (Lee *et al*., 1995), and CPD-Ae of yellow fever mosquito *Aedes aegypti* (Cho *et al*., 1991) also display the same properties with such substrates as they all conform to the required binding site composition. High activity with acidic P_1 residues could also be obtained if residue 312 is small and either residue 241 or residue 245 is basic. No naturally occurring serine carboxypeptidase with such activity or such a composition of the binding pocket has yet been found.

Attempts to co-crystallize CPD-Y with peptide derivatives to evaluate the effects of these mutations have not been successful. Hence, the mechanisms behind the elevated activities can only be speculated upon. It is noteworthy that increased activity with basic P_1 residues generally has been achieved by increases in k_{cat} while with acidic P_1 residues it has primarily been achieved by reductions in K_M. Thus, assuming that acylation is the rate-limiting step in the catalysis of these substrates ($k_2 \ll k_3$), as it is normally the case with serine proteases (Fersht, 1985), the observed increases in k_{cat} would correspond to equivalent increases in the rates of acylation while K_M under such condition becomes a measure of K_S. It follows that the low activity of CPD-Y with basic P_1 residues is due primarily to a low rate of acylation, presumably as a result of steric repulsion from the bulk of Trp312. When this bulk is reduced, the substrate can bind in a conformation that is more favorable for acylation. The additional introduction of a negative charge at position 241 or 245 may help attaining this conformation by providing a hydrophilic environment for the distal charge. In analogy, the low activity of CPD-Y with acidic P1 side chains is primarily due to poor binding of the substrate. Hence, reduction of the bulk of residue 312 and the introduction of a positive charge create an environment that better binds the negatively charged P_1 side chain. Apparently, the beneficial effects have been achieved without the formation of any salt bridges. Thus, it is conceivable that similar interactions could assist binding and catalysis with other enzymes that bind charged substrates on the water-accessible surface where salt bridges do not form.

The fact that optimal activity with charged P_1 residues was obtained by mutations at positions that were assigned to be outside of S_1 show that such interpretation has to take into consideration any alterations made at other positions in the binding pocket. In the presence of a Trp at position 312, the residues 241 and 245 are part of S_3 rather than S_1, but when residue 312 is small, these residues are part of both S_1 as well as S_3. These results emphasize the highly flexible nature of interactions between peptide side chains and the binding region of a protease.

REFERENCES

Becam, A.-M., Cullin, C., Grzybowska, E., Lacroute, F., Nasr, F., Ozier-Kalogeropoulos, O., Palucha, A., Slonimski, P. P., Zagulski, M., & Herbert, C. J. (1994) *Yeast 10*, 1−11.

Breddam, K. (1988) *Carlsberg Res. Commun. 53*, 309−320.

Cho, W. L., Deitsch, K. W., & Raikhel, A. S. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 10821−10824.

Dmochowska, A., Dignard, D., Henning, D., Thomas, D. Y., & Bussey, H. (1987) *Cell 50*, 573−584.

Dower, W. J., Miller, J. F., & Ragsdale, C. W. (1988) *Nucleic Acids Res. 16*, 6127−6145.

Endrizzi, J. A., Breddam, K., & Remington, S. J. (1994) *Biochemistry 33*, 11106−11120.

Fersht, A. (1985) in *Enzyme Structure and Mechanism*, 2nd ed., W. H. Freeman and Company, New York.

Gietz, D., St. Jean, A., Woods, R. A., & Schiestl, R. H. (1992) *Nucleic Acids Res. 20*, 1425.

Johansen, J. T., Breddam, K., & Ottesen, M. (1976) *Carlsberg Res. Commun. 41*, 1−14.

Kanstrup, A., & Buchardt, O. (1991) *Anal. Biochem. 194*, 41−44.

Kramer, B., Kramer, W., & Fritz, H.-J. (1984) *Cell 38*, 879−887.

Lee, B., Takeuchi, M., & Kobayashi, Y. (1995) *Curr. Genet. 27*, 159−165.

Lewis, M. K., & Thompson, D. V. (1990) *Nucleic Acids Res. 18*, 3439−3443.

Nielsen, T. L., Holmberg, S., & Petersen, J. G. L. (1990) *Appl. Microbiol. Biotechnol. 33*, 307−312.

Olesen, K., & Kielland-Brandt, M. C. (1993) *Protein Eng. 6*, 409−415.

Olesen, K., & Breddam, K. (1995) *Biochemistry 34*, 15689−15699.

Olesen, K., Mortensen, U. H., Aasmul-Olsen, S., Kielland-Brandt, M. C., Remington, S. J., & Breddam, K. (1994) *Biochemistry 33*, 11121−11126.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Schechter, I., & Berger, A. (1967) *Biochem. Biophys. Res. Commun. 27*, 157−162.

Schiestl, R. H., & Gietz, R. D. (1989) *Curr. Genet. 16*, 339−346.

Sherman, F. (1991) in *Methods in Enzymology*, Vol. 194, Guide to Yeast Genetics and Molecular Biology (Guthrie, C., Fink, C.R., Eds.) pp 3−21, Academic Press, Inc., New York.

Svendsen, I., Hofmann, T., Endrizzi, J., Remington, S. J., & Breddam, K. (1993) *FEBS Lett. 333*, 39−43.

Yanisch-Perron, C., Vieira, J., & Messing, J. (1985) *Gene 33*, 103−119.

Zell, R., & Fritz, H.-J. (1987) *EMBO J. 6*, 1809−1815.

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