Catalytic Efficiencies of Alkaline Proteinases from Microorganisms

Dessislava Georgieva[a], Nicolay Genov[b], Wolfgang Voelter[c], and Christian Betzel[a,*]

[a] Department of Biochemistry and Molecular Biology, University of Hamburg, Martin-Luther-King-Platz 6, 20146 Hamburg, Germany. Fax: +49-40-8998-4747. E-mail: Betzel@unisgi1.desy.de
[b] Institute of Organic Chemistry, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria
[c] Institute of Physiological Chemistry, University of Tübingen, Hoppe-Seyler-Str. 4, 72076 Tübingen, Germany

[*] Author for correspondence and reprint requests

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Catalytic efficiencies of proteinase K and mesentericopeptidase were determined using series of peptide-4-nitroanilide substrates and compared with those of subtilisin DY, savinase and esperase. For each enzyme the subsites S1–S4 were characterized. The data for the enzyme specificities were related to our high resolution X-ray models of the five enzymes and their complexes with peptides. The catalytic efficiencies of the alkaline proteinases are modulated by the hydrophobicity, solvent accessibility, flexibility and electrostatic effects in the substrate binding sites. The longer and nonpolar S1 loop offers more possibilities for hydrophobic interactions and increases the enzyme efficiency. S2 is a small narrow cleft which limits the possibilities for effective substitutions in P2. The wide specificity of S3 is due to its location on the protein surface of all investigated proteinases. The affinity of S4 for aromatic groups depends on the nature of the residues building the hydrophobic cavity.

Key words: Protease Efficiency, Enzyme Kinetic, Substrate Recognition, X-Ray Models

Introduction

Subtilisins and related enzymes (subtilases) as well as the intracellular proteinases are biocatalysts of outstanding scientific and biotechnological interest. These enzymes perform important biological functions connected with nutrition, activation of precursor proteins and cell differentiation (Perona and Craik, 1995). The ATP-dependent proteinases are involved in the regulation of biological processes through degradation of proteins in cells, including misfolded proteins (Gottesman, 1996). A new representative of this group of proteinases, termed CodWX, which differs from the other ATP-dependent proteolytic enzymes in its molecular architecture and activity, has been isolated from *Bacillus subtilis* and characterized (Kang *et al*., 2003). Recently, the crystal structure of a Lon protease, an ATP-dependent enzyme from *Methanococcus jannaschii* has been solved at 1.9 Å resolution (Im *et al*., 2004). The structure shows a unique catalytic site consisting of Ser-Lys-Asp residues, which considerably differs from the catalytic diad of other Lon proteinases (Im *et al*., 2004).

The possibilities for industrial production of large quantities and the commercial availability made possible a tremendous application of extracellular subtilases as biologically active components of washing powders and other cleaning materials, in the food processing industry, and in the medicine for preparation of proteolytic creams for treatment of wounds and collagen implants (Bott *et al*., 1996; Wolff *et al*., 1996; Nedkov, 1992). Subtilisin has been topically applied for therapeutic epidermal ablation (Fein *et al*., 2005). Bacterial proteinases with high stereo- and regiospecificity, acting under mild reaction conditions found application in the synthetic organic chemistry (Bordusa, 2002 and references therein). Enzymatic catalysis in organic solvents is an important area of research in bioorganic chemistry and biotechnology (Schmitke *et al*., 1994). Subtilases are broadly used in organic synthesis for enantio- and regiospecific reactions, amide bond syntheses and hydrolyses in both aqueous and organic solvents (Wang *et al*., 1997).

At present, many efforts are focused on improving of proteinase properties such as enzyme specificity and stability (Bordusa, 2002). The study of proteinase subsite specificity in relation to the respective X-ray model can provide important new insights into the mechanism of proteolytic action. Data in comparative aspect about the proteinase specificity and efficiency, obtained using the same

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experimental conditions, are limited. Usually, the kinetic data for the hydrolysis of synthetic substrates are collected at different conditions, which makes the results difficult for comparison and interpretation. In the present paper we report kinetic data about S1–S4 specificity and catalytic efficiency of proteinase K from the fungus *Tritirachium album Limber* and mesentericopeptidase from *Bacillus mesentericus*. The data are compared with those for savinase (Georgieva *et al*., 2001a), esperase (Georgieva *et al*., 2001b) and subtilisin DY (Georgieva *et al*., 2005). The kinetic data for all five proteinases were collected using the same experimental conditions. The enzymes were selected having in mind their biotechnological importance. Proteinase K possesses a very high proteolytic activity and found application in the preparation of nucleic acids (Wiegers and Hilz, 1971). This enzyme digests even keratin (Betzel *et al*., 1988). Proteinase K is widely used in clinics and laboratories to distinguish between the normal and infectious forms of prions, proteins whose structural transformation leads to invariantly fatal neurodegenerative diseases like "mad cow disease" (Prusiner, 1998). Savinase and esperase are closely related proteinases from *Bacillus lentus* with greater thermal stability and higher alkaline performance than "classical" subtilisins (Novo Product No 219, available from Novo Nordisk A/S Bagsvaerd, Denmark). These proteinases are detergent compatible and thermostable at highly alkaline pH in the region 8–12. They are widely used as detergent additives in cleaning materials. Subtilisin DY, from a X-ray irradiated strain of *Bacillus licheniformis* var. DY, was used as a component of the laundry powder Biopon and for the preparation of proteolytic creams and collagen implants for the surgery (Nedkov, 1992). Mesentericopeptidase is also perspective for biotechnological purposes. The kinetic data were related to the crystallographic models of the five proteinases and their complexes with synthetic peptides using our X-ray coordinates. Conclusions about the role of the hydrophobicity, structural flexibility, electrostatic interactions and solvent accessibility for the specificity and catalytic efficiency of the proteinase active site are made.

Materials and Methods

Enzymes

Proteinase K was purchased from Sigma Chemical (St. Louis). Mesentericopeptidase was isolated and purified as described by Karadjova *et al*. (1970). The enzyme was precipitated by saturation of the culture liquid of *Bacillus mesentericus* with ammonium sulphate at a content between 25 and 38%. The sediment was further fractionated by chromatography on carboxymethyl cellulose using 5×10^{-3} M Tris [tris(hydroxymethyl)aminomethane]-HCl buffer, pH 7.2, and a salt gradient of 0–0.4 M NaCl at 4 °C. Fractions containing mesentericopeptidase were collected and subjected to force-dialysis. At the end of this process the enzyme crystallized which is an additional indication of homogeneity. Before the experiments the two proteinases as well as subtilisin DY, esperase and savinase were purified additionally by gel-chromatography to separate autolytic products. The enzymes used for the kinetic measurements were homogeneous by gel-electrophoresis. The sources of the three enzymes used before for investigation of their specificity and compared kinetically with proteinase K and mesentericopeptidase are as follows: subtilisin DY was isolated from the strain DY of *Bacillus licheniformis* as described by Genov *et al*. (1982). Esperase and savinase, secreted by the alkalophilic bacterium *Bacillus lentus*, were kindly supplied by Novo Nordisk A/S Bagsvaerd, Denmark.

Substrates

Synthetic peptide-4-nitroanilide substrates with the general structures Suc-Ala-Ala-Pro-Aa-4NA (Aa = Ala, Leu, Nle, Val, Phe, Glu, Lys), Suc-Phe-Pro-Phe-4NA, Suc-Phe-Leu-Phe-4NA, Suc-Ala-Ala-Phe-4NA, Suc-Gly-Gly-Phe-4NA, Suc-Ala-Aa-Pro-Phe-4NA (Aa = Gly, Ala, Leu, Phe, Trp, Glu) and Suc-Phe-Ala-Ala-Phe-4NA were purchased from Bachem (Heidelberg, Germany). All chemicals and reagents used were of analytical grade.

Kinetic measurements

Kinetic experiments with synthetic peptide-4-nitroanilide substrates were carried out in 0.1 M Tris-HCl buffer, pH 8.2, at 25 °C and in the presence of 5% *N,N*-dimethylformamide (DMFA). The release of 4-nitroaniline was monitored spectrophotometrically at 405 nm (ε_{405} = 9600 l M^{-1} cm^{-1}). The enzyme concentration was usually in the range 1.95×10^{-8}–9.12×10^{-9} M and that of the substrate varied between 1.6×10^{-3} and 1.2×10^{-4} M. Kinetic parameters were calculated from

initial rate measurements of the substrate hydrolysis using a nonlinear regression analysis.

Computer graphic studies

Computer graphic studies of the five proteinase structures were carried out on a Silicon Graphics O_2 workstation using the program TURBO-FRODO (Roussel and Cambillau, 1991).

Results and Discussion

Catalytic efficiencies of proteinase K and mesentericopeptidase

The S1 specificity is of particular importance because it was shown that this subsite, together with S4, dominates the substrate preference (McPhalen *et al.*, 1985). The S1 preferences of proteinase K and mesentericopeptidase were investigated by a series of seven peptide substrates, containing short, long or branched aliphatic side chains, aromatic or oppositely charged groups in position P1 (Tables I–III). The substrate preference is determined by the specificity constant k_{cat}/K_m. In these substrates only a single residue was varied, that in P1. Both enzymes exhibit marked preference for an aromatic or long aliphatic side chain in this position as it is evident from the values of k_{cat}/K_m shown in Table III. The small methyl group of alanine is less favourable for catalysis. The charged side chains of glutamic acid and lysine as well as the β-branched functional group of valine are very poorly accepted by S1, Suc-Ala-Ala-Pro-Val-4NA being the worst substrate. However, the efficiency towards the positively charged lysyl substrate is almost one order of magnitude higher than that exhibited with the substrate containing Glu in P1 which means that electrostatic effects are also important for the catalysis. The high efficiencies of the two proteinases towards the first three substrates, shown in Table III, are derived from the lower Michaelis constants (high affinity) (Table I) and higher catalytic constants (turnover number) (Table II). The low efficiency observed for the substrate with Ala in position P1 is due mainly to the low catalytic constant (Table II). The accommodation of glutamic acid and lysine at S1 is more difficult which reflects in the considerably higher K_m values. The turnover of these substrates is also very low. The side chain of valine in P1 is the most poorly accommodated functional group. The highest Michaelis constant of 5.0 mM and the lowest catalytic constant of (0.30 ± 0.02) s^{-1} were calculated for the enzyme hydrolysis of Suc-Ala-Ala-Pro-Val-4NA (Tables I and II). With Proteinase K, the following decreasing order of catalytic efficiency was observed: Phe > Nle > Leu >> Ala

Table I. Michaelis constants for the hydrolysis of synthetic peptidyl substrates by proteinases from microorganisms.

P_5 P_4 P_3 P_2 $P_1 \downarrow P_1'$	Proteinase K K_m [mM]	Mesentericopeptidase K_m [mM]	Subtilisin DY[a] K_m [mM]	Savinase[b] K_m [mM]	Esperase[c] K_m [mM]
Suc-Ala-Ala-Pro-Phe-4NA	0.23 ± 0.02	0.28 ± 0.02	0.17 ± 0.01	0.20 ± 0.02	0.17 ± 0.02
Suc-Ala-Ala-Pro-Leu-4NA	0.20 ± 0.01	0.29 ± 0.02	0.17 ± 0.01	0.56 ± 0.04	0.61 ± 0.04
Suc-Ala-Ala-Pro-Nle-4NA	0.24 ± 0.02	0.23 ± 0.02	0.26 ± 0.02	0.67 ± 0.05	0.35 ± 0.02
Suc-Ala-Ala-Pro-Ala-4NA	0.28 ± 0.02	0.22 ± 0.01	0.43 ± 0.03	0.61 ± 0.04	0.45 ± 0.03
Suc-Ala-Ala-Pro-Glu-4NA	1.05 ± 0.06	1.43 ± 0.10	1.33 ± 0.07	1.43 ± 0.11	0.49 ± 0.03
Suc-Ala-Ala-Pro-Lys-4NA	1.25 ± 0.07	0.62 ± 0.05	0.58 ± 0.03	1.53 ± 0.12	0.72 ± 0.05
Suc-Ala-Ala-Pro-Val-4NA	5.0 ± 0.6	0.42 ± 0.03	0.40 ± 0.02	8.0 ± 0.7	10.0 ± 0.9
Suc-Phe-Pro-Phe-4NA	0.32 ± 0.03	0.45 ± 0.03	–	0.42 ± 0.03	0.37 ± 0.03
Suc-Phe-Leu-Phe-4NA	1.43 ± 0.08	1.62 ± 0.12	–	1.43 ± 0.11	1.67 ± 0.12
Suc-Ala-Ala-Phe-4NA	0.55 ± 0.04	0.72 ± 0.05	0.61 ± 0.04	0.30 ± 0.02	0.80 ± 0.07
Suc-Gly-Gly-Phe-4NA	1.00 ± 0.05	2.50 ± 0.10	0.56 ± 0.04	2.86 ± 0.3	5.55 ± 0.60
Suc-Ala-Phe-Pro-Phe-4NA	0.33 ± 0.03	0.35 ± 0.02	0.36 ± 0.02	0.18 ± 0.02	0.12 ± 0.01
Suc-Ala-Ala-Pro-Phe-4NA	0.23 ± 0.02	0.28 ± 0.03	0.17 ± 0.01	0.20 ± 0.02	0.17 ± 0.02
Suc-Ala-Trp-Pro-Phe-4NA	0.17 ± 0.01	0.36 ± 0.03	0.36 ± 0.02	0.83 ± 0.05	0.32 ± 0.02
Suc-Ala-Gly-Pro-Phe-4NA	0.22 ± 0.02	0.24 ± 0.02	0.19 ± 0.01	0.30 ± 0.02	0.17 ± 0.01
Suc-Ala-Leu-Pro-Phe-4NA	0.20 ± 0.01	0.21 ± 0.02	0.20 ± 0.01	0.22 ± 0.02	0.12 ± 0.01
Suc-Ala-Glu-Pro-Phe-4NA	0.29 ± 0.02	0.20 ± 0.01	0.21 ± 0.01	0.70 ± 0.05	0.91 ± 0.06
Suc-Phe-Ala-Ala-Phe-4NA	0.01 ± 0.001	0.02 ± 0.001	0.02 ± 0.001	0.10 ± 0.01	0.05 ± 0.01

[a] Data from Georgieva *et al.* (2005).
[b] Data from Georgieva *et al.* (2001a).
[c] Data from Georgieva *et al.* (2001b).

Table II. Catalytic constants for the hydrolysis of synthetic peptidyl substrates by proteinases from microorganisms.

P_5 P_4 P_3 P_2 $P_1 \downarrow P_1'$	Proteinase K k_{cat} [s^{-1}]	Mesentericopeptidase k_{cat} [s^{-1}]	Subtilisin DY[a] k_{cat} [s^{-1}]	Savinase[b] k_{cat} [s^{-1}]	Esperase[c] k_{cat} [s^{-1}]
Suc-Ala-Ala-Pro-Phe-4NA	178.0 ± 5.0	151.3 ± 4.8	108.2 ± 2.6	26.9 ± 0.9	31.2 ± 1.0
Suc-Ala-Ala-Pro-Leu-4NA	43.2 ± 1.6	153.6 ± 4.8	53.9 ± 1.8	64.7 ± 3.0	68.4 ± 3.1
Suc-Ala-Ala-Pro-Nle-4NA	84.4 ± 3.2	62.6 ± 2.0	40.0 ± 1.7	18.2 ± 0.6	31.2 ± 1.1
Suc-Ala-Ala-Pro-Ala-4NA	7.3 ± 0.30	5.00 ± 0.18	2.9 ± 0.1	19.3 ± 0.6	30.0 ± 1.0
Suc-Ala-Ala-Pro-Glu-4NA	0.9 ± 0.04	1.00 ± 0.04	0.5 ± 0.03	0.32 ± 0.02	1.07 ± 0.05
Suc-Ala-Ala-Pro-Lys-4NA	16.9 ± 0.6	2.28 ± 0.09	1.1 ± 0.05	0.15 ± 0.01	0.86 ± 0.05
Suc-Ala-Ala-Pro-Val-4NA	0.30 ± 0.02	0.07 ± 0.003	0.3 ± 0.02	0.9 ± 0.05	0.60 ± 0.04
Suc-Phe-Pro-Phe-4NA	0.30 ± 0.02	0.25 ± 0.02	–	0.11 ± 0.02	0.08 ± 0.01
Suc-Phe-Leu-Phe-4NA	3.9 ± 0.2	2.9 ± 0.1	–	1.2 ± 0.05	1.10 ± 0.05
Suc-Ala-Ala-Phe-4NA	17.0 ± 0.6	15.2 ± 0.5	57.0 ± 2.0	20.2 ± 0.6	20.9 ± 0.6
Suc-Gly-Gly-Phe-4NA	3.5 ± 0.1	2.2 ± 0.1	12.5 ± 0.4	3.0 ± 0.1	1.1 ± 0.04
Suc-Ala-Phe-Pro-Phe-4NA	145.1 ± 4.5	91.4 ± 2.9	87.5 ± 3.0	38.2 ± 1.1	31.2 ± 1.0
Suc-Ala-Ala-Pro-Phe-4NA	178.0 ± 5.0	151.3 ± 4.8	108.2 ± 2.6	26.9 ± 0.9	31.2 ± 1.0
Suc-Ala-Trp-Pro-Phe-4NA	175.3 ± 5.0	91.6 ± 2.9	120.2 ± 5.0	54.0 ± 2.2	48.0 ± 1.9
Suc-Ala-Gly-Pro-Phe-4NA	66.2 ± 2.5	43.4 ± 1.4	59.0 ± 2.2	15.0 ± 0.5	16.4 ± 0.5
Suc-Ala-Leu-Pro-Phe-4NA	191.4 ± 5.4	71.4 ± 2.5	89.3 ± 3.5	43.4 ± 1.8	10.2 ± 0.4
Suc-Ala-Glu-Pro-Phe-4NA	231.2 ± 6.5	142.4 ± 4.5	134.0 ± 4.0	70.0 ± 2.8	69.6 ± 2.8
Suc-Phe-Ala-Ala-Phe-4NA	55.0 ± 1.6	59.2 ± 2.0	86.2 ± 3.1	217.0 ± 5.0	161.3 ± 3.7

[a] Data from Georgieva *et al.* (2005).
[b] Data from Georgieva *et al.* (2001a).
[c] Data from Georgieva *et al.* (2001b).

> Lys >> Glu >> Val. With mesentericopeptidase, the order is Phe = Leu > Nle >> Ala >> Lys >> Glu >> Val. The S1 specificity of savinase (Table III), obtained by peptide nitroanilides, is in good agreement with that determined by internally quenched fluorescent substrates (Grøn *et al*., 1992). One difference is that the authors mentioned above found a preference for Phe over Leu while, according to our data, the S1 specificity for Phe is practically the same as that for Leu. Also, we have included substrates with norleucine and glutamic acid in P1.

Substitution of Ala by Gly in positions P2 and P3 of the tripeptide nitroanilides leads to a decrease of the affinity and catalytic efficiency of proteinase K and mesentericopeptidase (Tables I and III). In the case of the tetrapeptide substrates the same substitution in P3 did not change K_m (Table I). Consequently, the reason for the observed difference in the enzyme affinity is not the substitution in P3 but that in P2. The preferences of the both proteinases for the residue in position P2, according to the values of k_{cat}/K_m, are: Leu > Pro and Ala > Gly.

All substrates, used for the determination of the P3 specificity, were efficiently hydrolyzed by the two enzymes. The differences in the catalytic parameters were not big. Proteinase K prefers Trp in P3 and the lowest K_m value was observed with Suc-Ala-Trp-Pro-Phe-4NA as a substrate (Table I). Substitution of Trp for Leu, Gly and Ala slightly changed the Michaelis constant. The highest k_{cat} was calculated for the hydrolysis of Suc-Ala-Glu-Pro-Phe-4NA while the values for the other substrates, except that with Gly in P3, were of the same magnitude (Table II). In the same time, mesentericopeptidase exhibits the lowest affinity (the highest K_m value) towards the substrate with aromatic side chains like Trp and Phe in P3 (Table I). The highest turnover numbers were observed with the substrates Suc-Ala-Ala-Pro-Phe-4NA and Suc-Ala-Glu-Pro-Phe-4NA (Table II). The decreasing order of P3 specificity for proteinase K is Trp > Leu > Glu = Ala > Phe > Gly and that for mesentericopeptidase is Glu > Ala > Leu > Trp = Phe > Gly (Table III).

The most favourable substrate for proteinase K and mesentericopeptidase was Suc-Phe-Ala-Ala-Phe-4NA, with Phe in P4. The high efficiency is derived from greater binding (the K_m values are an order of magnitude lower in comparison to those for all other substrates) (Table I). Evidently, S4 has an extremely high affinity for the aromatic phenyl group. Such a preference has been observed also for other proteinases (Grøn *et al*., 1992). The efficiency of proteinase K towards this

Table III. Specificity constants (catalytic efficiency) for the hydrolysis of synthetic peptidyl substrates by proteinases from microorganisms.

P_5 P_4 P_3 P_2 $P_1 \downarrow P_1'$	Proteinase K k_{cat}/K_m [s^{-1} мМ$^{-1}$]	Mesentericopeptidase k_{cat}/K_m [s^{-1} мМ$^{-1}$]	Subtilisin DY[a] k_{cat}/K_m [s^{-1} мМ$^{-1}$]	Savinase[b] k_{cat}/K_m [s^{-1} мМ$^{-1}$]	Esperase[c] k_{cat}/K_m [s^{-1} мМ$^{-1}$]
Suc-Ala-Ala-Pro-Phe-4NA	782 ± 90	544 ± 56	640 ± 52	134 ± 16	184 ± 24
Suc-Ala-Ala-Pro-Leu-4NA	217 ± 19	533 ± 53	318 ± 30	115 ± 12	112 ± 12
Suc-Ala-Ala-Pro-Nle-4NA	355 ± 43	275 ± 33	155 ± 18	27 ± 3	89 ± 7
Suc-Ala-Ala-Pro-Ala-4NA	26 ± 3	23 ± 2	6.8 ± 0.7	32 ± 3	67 ± 6
Suc-Ala-Ala-Pro-Glu-4NA	0.86 ± 0.09	0.68 ± 0.05	0.38 ± 0.04	0.22 ± 0.04	2.2 ± 0.2
Suc-Ala-Ala-Pro-Lys-4NA	13.6 ± 1.2	3.71 ± 0.45	1.91 ± 0.19	0.10 ± 0.02	1.2 ± 0.1
Suc-Ala-Ala-Pro-Val-4NA	0.06 ± 0.01	0.17 ± 0.02	0.76 ± 0.09	0.11 ± 0.02	0.06 ± 0.01
Suc-Phe-Pro-Phe-4NA	0.95 ± 0.15	0.52 ± 0.04	–	0.26 ± 0.06	0.21 ± 0.04
Suc-Phe-Leu-Phe-4NA	2.75 ± 0.30	1.8 ± 0.2	–	0.84 ± 0.10	0.66 ± 0.08
Suc-Ala-Ala-Phe-4NA	31.2 ± 3.4	22.7 ± 0.7	94 ± 9	67 ± 6	26 ± 3
Suc-Gly-Gly-Phe-4NA	3.5 ± 0.3	0.88 ± 0.03	22 ± 2	1.05 ± 0.05	0.20 ± 0.02
Suc-Ala-Phe-Pro-Phe-4NA	445 ± 54	263 ± 23	244 ± 22	212 ± 27	260 ± 30
Suc-Ala-Ala-Pro-Phe-4NA	782 ± 90	544 ± 56	640 ± 52	134 ± 16	184 ± 24
Suc-Ala-Trp-Pro-Phe-4NA	1036 ± 90	257 ± 29	335 ± 32	65 ± 7	150 ± 16
Suc-Ala-Gly-Pro-Phe-4NA	304 ± 39	183 ± 21	312 ± 28	50 ± 5	96 ± 10
Suc-Ala-Leu-Pro-Phe-4NA	961 ± 75	344 ± 45	448 ± 40	197 ± 26	85 ± 10
Suc-Ala-Glu-Pro-Phe-4NA	802 ± 78	715 ± 58	640 ± 50	100 ± 12	76 ± 9
Suc-Phe-Ala-Ala-Phe-4NA	5572 ± 717	2972 ± 249	4328 ± 377	2170 ± 200	3226 ± 600

[a] Data from Georgieva *et al.* (2005).
[b] Data from Georgieva *et al.* (2001a).
[c] Data from Georgieva *et al.* (2001b).

substrate is two times higher than that of mesentericopeptidase (Table III).

Comparison of kinetic parameters for the five proteinases from microorganisms

Kinetic data for five proteinases from microorganisms and of biotechnological importance are compared in Tables I–III. The data collection was made at the same experimental conditions. This makes the calculated parameters comparable. Regardless of some differences in the kinetic constants for the same substrates, general tendencies are evident. A marked preference of S1 for the aromatic group of Phe is a common feature of the investigated proteinases. The next very well accepted by this subsite residue is Leu. Mesentericopeptidase and savinase have practically the same specificity constants for the substrates with Phe or Leu in P1. Nle is relatively well accepted while S1 does not tolerate the methyl group of Ala and the long charged side chains of Glu and Lys. The poorest substrate is that with Val in P1. The inefficiency in the hydrolysis of substrates with Glu, Lys and Val is common to all five proteinases. Only in the case of subtilisin DY there is some preference for Val over Glu or Lys. The S2 subsite definitely exhibits a preference for Ala over Gly and for Leu over Pro. The enzymes discriminate moderately among the P3 residues and their S3 subsites are very flexible with respect to the accommodation of different amino acid side chains. Some proteinases (proteinase K, esperase, savinase) exhibit a preference for aromatic side chains in P3, like those of Trp and Phe, but the other two (mesentericopeptidase and subtilisin DY) prefer Glu in this position. Gly in P3 is not effective for the catalysis. The five enzymes exhibit a marked preference for the aromatic group of Phe in P4 (Table III) and in all cases the high efficiency, which drastically differs from those observed with the other substrates, is derived from greater binding (Table I).

Relation of the kinetic data to the X-ray models

The three-dimensional models of the five proteinases were built using our X-ray coordinates obtained by synchrotron radiation at DESY-Hamburg (Betzel *et al.*, 1988, 1992, 1996; Dauter *et al.*, 1991; Eschenburg *et al.*, 1998). We have used also our crystallographic data about complexes of the investigated enzymes with peptide inhibitors.

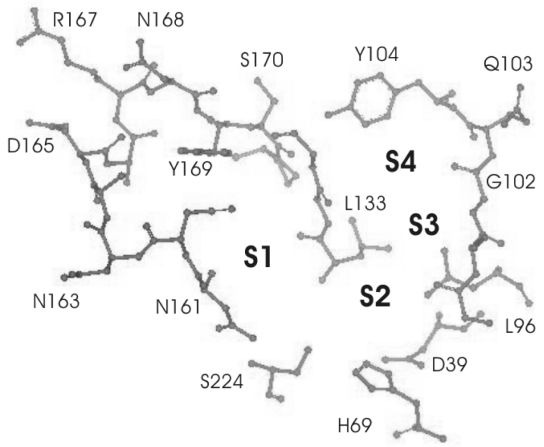

Fig. 1. View of the S1–S4 substrate binding site of proteinase K (PDB code 1PEK).

Fig. 1 shows the S1–S4 region of substrate binding in proteinase K (PDB code 1PEK). The substrate recognition sites in the five enzymes are made up by two nearly parallel peptide sequences including residues 99–104 on one side and 125–131 on the other. These regions are moderately accessible to the solvent. They flank the residues P1 to P3 of the peptide substrate and form an antiparallel β-pleated sheet with it during catalysis. Most of the side chains forming the P1 loop point away from the centre of the loop and in this way a wide cavity is formed, capable to accommodate different amino acid residues. This is in line with the broad specificity of the investigated proteinases which can hydrolyse peptide substrates with aromatic, aliphatic or charged side chains in P1. S1 is hydrophobic in all cases, which explains the preference for nonpolar residues over those with electrostatically charged groups. For this reason the proteinases exhibit very low catalytic efficiency towards peptide nitroanilides with Lys or Glu in P1 (Table III). Proteinase K, mesentericopeptidase and subtilisin DY prefer definitely Lys over Glu, which suggests a negatively charged microenvironment of the respective site. An opposite effect was observed with esperase which is due probably to the presence of the positively charged guanidine group of Arg158 in S1. The five crystallographic structures show that the entrance of the S1 subsite is too narrow to accommodate the β-branched side chain of Val and the respective peptide nitroanilide is the worst substrate for the enzymes whose catalytic efficiencies are compared in Table III. Such limitation has also been suggested for subtilisin BPN' (Takeuchi *et al*., 1991).

Comparison of the P1 substrate binding loops of the five proteinases showed higher hydrophobicity of the S1 subsites in proteinase K, subtilisin DY and mesentericopeptidase due to the presence of more hydrophobic side chains in comparison to the respective sites in savinase and esperase. Also, the S1 loop in the last two enzymes is four residues shorter which reduces the possibilities for hydrophobic contacts. The differences in the nature of the side chains building S1 as well as those in the geometry of the S1 subsites can explain the higher catalytic efficiencies of the first three enzymes for substrates with hydrophobic P1 residues. The higher affinity of proteinase K, subtilisin DY and mesentericopeptidase for aliphatic P1 residues reflects in the considerably lower K_m values which are more than 2 times lower than those for esperase and savinase (Table I). This is due probably to the fewer possibilities for hydrophobic contacts in the shorter P1 loop of both enzymes.

S2 is the smallest subsite and represents a narrow and shallow "cleft" formed by residues Leu96, His64 and Gly100 (numbering according to the sequences of subtilisin DY and mesentericopeptidase). These residues are all conserved in the five proteinases. It seems that steric effects are important for the interaction of P2 with S2. The higher K_m value for the substrate with Leu in P2 (Table I) is probably due to more difficult accommodation of the longer aliphatic chain of this residue. S2 can also accommodate Pro. The preference of the subsite for Leu over Pro can be explained with the X-ray model of the complex between proteinase K and a peptide substrate with Pro in P2 (PDB code 1 PEK) which shows that this residue is involved only in weak van der Waals contacts with Gly100. The preference for Ala in P2 over Gly is due to the possibility for the methyl group to participate in hydrophobic interactions with S2 while Gly lacks a side chain. The geometry of S2 makes effective variations of residues in P2 more difficult in comparison to the other subsites.

S3 can not be defined as a "cleft" or "pocket". The P3 residue of the peptide substrates lies on the protein surface and even the existence of S3 is under question. The side chain of P3 should be directed to the solvent and this location can explain the broad S3 specificity.

The subsite S4, as S1, is very important for the proteinase specificity. The binding to this site con-

tributes significantly to the catalytic efficiency. S4 is a hydrophobic pocket formed by the side chains of several residues: that in position 104, which is Tyr, Val or Leu in the investigated proteinases, the residue in position 107, which is Ile in proteinase K, mesentericopeptidase, subtilisin DY and savinase, and Val in esperase, as well as by Asn142 in proteinase K or by the conserved in the other four proteinases Leu135. The segment of residues 128–131 also participates in the formation of the subsite. The S4–P4 interactions are dominated by hydrophobic forces. S4 in the five proteinases exhibits an extremely high catalytic efficiency for the peptide substrate with Phe in P4 (Table III). Similar preference has been observed with other proteinases (Grøn *et al*., 1992 and references therein). The efficiency is derived from the very high affinity of this subsite for the aromatic phenol ring (Table I). The substitution of Tyr104 in proteinase K, mesentericopeptidase and subtilisin DY by Val104 in savinase or Leu104 in esperase leads to considerable decrease of the enzyme affinity for the peptide substrate with Phe in P4 which reflects in increased K_m values (Table I). The highest K_m value was observed with savinase. This is due probably to the additional rigidity of S4 in this enzyme imposed by the two prolines in positions 129 and 131 which prevents the best accommodation of the substrate. The lower subsite flexibility also reflects in the catalytic efficiency towards Suc-Phe-Ala-Ala-Phe-4NA which in the case of savinase is the lowest one in comparison with the other four enzymes (Table III). The other four proteinases have Gly (mesentericopeptidase, esperase and subtilisin DY) or Ser (proteinase K) in position 131. Evidently, the presence of an aromatic group in S4 is more favourable for the interactions with the phenyl group of the substrate. The X-ray model of the complex between proteinase K and the peptide inhibitor with Phe in P4 (PDB code 1PEK) showed that both segments of the substrate recognition site are flexible and move appreciably to accommodate the inhibitor. The aromatic group of $Phe4^I$ (I means inhibitor) fills very well the hydrophobic S4 pocket which seems to be designed for a preferable binding of a phenyl ring. The carbonyl group of $Phe4^I$ is fixed by two hydrogen bonds with Asn161 (N_δ) and Thr223 (O_γ). The phenyl ring of $Phe4^I$ can also interact with the aliphatic side chains of Val104 (savinase) or Leu104 (esperase) but the affinity for the respective subsites is not so high.

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