The Topographical Differences in the Active Site Region of α-Chymotrypsin, Subtilisin Novo, and Subtilisin Carlsberg. Mapping the Aromatic Binding Site by Inhibitors (Virtual Substrates)†

Hans Rudolf Bosshard* and Arieh Berger‡

ABSTRACT: Peptides of the structure benzyloxycarbonyl-L-alanyl-L-alanyl-L-P_1 are competitive inhibitors (virtual substrates) of the hydrolysis of ester substrates by α-chymotrypsin, subtilisin Novo, and subtilisin Carlsberg. P_1 stands for a series of 19 different L-amino acids and derivatives thereof. Inhibition constants, $\bar{K}_i$, and standard free energies of binding, $\Delta F°$, were determined from inhibition experiments. Comparison of $\bar{K}_i$ and $\Delta F°$ values revealed pronounced differences in the binding of hydrophobic side chains to the specificity determining subsite S_1 of the three enzymes, α-chymotrypsin delicately differentiating variously substituted phenylalanines and geometrical isomers of aryl side chains in P_1 but the subtilisins accommodating almost any aromatic residue. S. Carlsberg binds most inhibitors more strongly than does S. Novo by about −1 kcal/mol in $\Delta F°$. An outstanding exception is *S*-diphenylmethylcysteine in P_1 which binds well only to S. Novo. Evidence for differences in the three-dimensional structure of the active site region of S. Novo and S. Carlsberg is presented and discussed in the light of kinetic as well as of X-ray and sequence data.

The interaction of enzymes with specific substrates or inhibitors is an extremely useful model for studying the features and essentials of biological recognition. Hydrolytic enzymes are especially adequate for this purpose. Being abundant and seemingly uniform in their catalytic performances, they are highly diversified with regard to their substrate specificities. Several proteolytic enzymes were found to have quite extended binding sites which contribute on the one hand to their common task, namely binding peptide chains and breaking peptide (or ester) bonds, and which on the other hand distinguish their individual substrate specificities (Schechter and Berger, 1967; Abramowitz *et al.*, 1967; Berger and Schechter, 1970; Berger *et al.*, 1971; Atlas and Berger, 1972; Segal *et al.*, 1971; Segal, 1972; Robertus *et al.*, 1972a,b).

Since recognition involves as a first step an interaction of two molecules, it is best measured quantitatively in terms of a binding constant which is directly related to the standard free energy of binding by $\Delta F° = -RT \ln \bar{K}_i$. It is for this reason that the interaction of reversible inhibitors with enzymes is an attractively simple model. In contrast, the "substrate approach" is fraught with difficulties involving perturbations arising from the kinetics and mechanism of the catalytic reaction. However, another problem arises. If data from inhibitor binding are to yield information relevant to the enzyme-catalyzed reaction, the inhibitors have to be chosen so as to resemble real substrates as close as possible. In addition, the chosen inhibitors should guarantee a unique and uniform binding mode which again should approximate the binding of substrates to the enzymes active site. In the case of proteases, short peptide acids, *e.g.*, Z-Ala-Ala-Phe,[1] fulfill these requirements for two reasons. They are themselves products of the enzymic action on a larger substrate, say Z-Ala-Ala-Phe-Ala cleaved specifically by CT, and may therefore be regarded as virtual substrates. Secondly, they display multipoint attachment to an enzyme's extended binding site which is essential for strong binding and, even more important, for uniqueness of binding. Recent findings of nonproductive binding of small substrate molecules to CT and papain stress the importance of a unique binding mode whenever meaningful and comparable data are to be deduced from such studies (Hinkle and Kirsch, 1971; Fastrez and Fersht, 1973). Extensive use of peptide inhibitors has been made in elucidating the subsites of papain (Berger *et al.*, 1971).

In the work reported here, we have applied the "inhibitor approach" to the study of the hydrophobic binding sites of CT, S. Novo, and S. Carlsberg. The amino acid sequence of CT shows no obvious sequence homology with the two subtilisins (Smith *et al.*, 1968). In contrast to this finding, Kraut and coworkers revealed by elegant X-ray work a striking conformational homology between the active sites of CT and S. Novo[2] (Kraut, 1971; Robertus *et al.*, 1972a,b; Wright,

† From the Department of Biophysics, The Weizmann Institute of Science, Rehovot, Israel. *Received June 12, 1973.* Part of the data on α-chymotrypsin was presented at the 9th International Congress of Biochemistry, Stockholm, July 1973.

* Recipient of a fellowship from the Schweizerischer Nationalfonds zur Förderung der wissenschaftlichen Forschung. Present address: MRC Laboratory for Molecular Biology, Hills Road, Cambridge CB2 2QH, England.

‡ Deceased November 1, 1972.

[1] Abbreviations according to IUPAC-IUB rules, "Symbols for Amino Acid Derivatives and Peptides, Recommendations (1971)," see, *e.g.*, *J. Biol. Chem. 247*, 977 (1972). In particular, the following abbreviations have been used: Z, benzyloxycarbonyl; Ac, acetyl; Succ, succinyl; Bzl, benzyl; Bz, benzoyl; Acm, acetamidomethyl; Dpm, diphenylmethyl; −OEt, ethoxy; −OMe, methoxy; −ONSu, succinimidooxy. Additional abbreviations are: CT, α-chymotrypsin; S., subtilisin DMF, *N,N*-dimethylformamide, TCI, *N-trans*-cinnamoylimidazole; Ala(1Nph), β-(1-naphthyl)alanine; Ala(2Nph), β-(2-naphthyl)alanine; Ala(6Qui), β-(6-quinolyl)alanine. Phe(4F), Phe($2CH_3$) etc., ring-substituted phenylalanines, nature and position of substituents are given in the parentheses. Phe(F_5), (*ar*-pentafluoro)phenylalanine. All amino acids are of the L configuration.

[2] The cited work was actually done on S. BPN′ which is identical with S. Novo in its primary (Markland and Smith, 1971) and crystal structure (Robertus *et al.*, 1971).

1972). Thus, although CT and the subtilisins belong to two different classes of serine proteases (Hartley, 1970), the enzymes converged in a practically identical catalytic apparatus, *viz.* the charge-relay system of the catalytic sites (Robertus *et al.*, 1972b; Wright, 1972), and in fairly similar substrate specificities (Morihara *et al.*, 1969; Pattabiraman and Lawson, 1972). *How extensive is this convergency as regards shape and dimension of the specificity determining hydrophobic binding site?*

S. Carlsberg and S. Novo are sequence homologous enzymes. S. Carlsberg differs from S. Novo in only 84 out of 275 amino acid positions and in a deletion in position 56 (Smith *et al.*, 1968). The three-dimensional (3-D) structure of S. Carlsberg is not known. Based on the evolutionary convergence of CT and S. Novo and on the sequence homology between S. Novo and S. Carlsberg, it has been tentatively anticipated that the two subtilisins have a very similar 3-D structure with probably an identical active site region (Wright *et al.*, 1969). Barel and Glazer (1968) concluded from kinetic experiments that S. Novo and S. Carlsberg are "qualitatively indistinguishable from the standpoint of substrate specificity" but that "significant quantitative differences" exist in the catalytic constants (K_m(app), k_{cat}) for some substrates. Evidence for subtle but distinct differences in the geometry of the two active sites were deduced from the different inhibition behavior of N^{α}-Bz-Arg toward the two enzymes (Bosshard, 1973a). *Are the differences in substrate specificities confined to minor fluctuations of K_m(app) and k_{cat} for comparable substrates, or are there any topographical differences between these two sequence homologous enzymes in at least part of their active sites?*

Niemann and coworkers published a long list of competitive inhibitors of CT (Foster and Niemann, 1955). Elucidating the binding of small, reversible inhibitors, some of them pseudosubstrates, to CT has since been a popular endeavor, and various physical techniques have been applied to the problem (see, *e.g.*, Bernhard *et al.*, 1966; Johnson and Knowles, 1966; McClure and Edelman, 1967; Gammon *et al.*, 1972). Reports about inhibitor binding to subtilisins have been less numerous. Outstanding is the very recent X-ray work of Kraut's group who meticulously described the binding of virtual substrates, *e.g.*, of Z-Gly-Gly-Tyr, to S. Novo, culminating in a highly attractive speculation about the enzymes stereochemical mechanism of action (Robertus *et al.*, 1972b).

In the following we present results from kinetic studies about the binding to CT, S. Novo, and S. Carlsberg of inhibitors of the general structure Z-Ala-Ala-P_1,[3] where P_1 stands for various L-amino acids. The findings at least partly answer the two questions asked above.

Materials and Methods

Enzymes. α-Chymotryspin, three times crystallized, lyophilized, and salt free, was from Worthington Biochemical Corporation (Freehold, N. J.), batch CD1 2DC. The enzyme was used without further purification. Its operational normality was determined by titration with TCI according to Schonbaum *et al.* (1961), and was found to be 88%. S. Carlsberg (lot 70-3) and S. Novo (lot 120-2) were from Novo Industries, Copenhagen. The two enzymes in a further purified form (chromatographed consecutively on Sephadex G-25 in 0.01 M phosphate (pH 7.0) and on CM-cellulose, gradient of 0.01–0.1 M phosphate (pH 7.0)) were kindly supplied by Dr. R. S. Roche. The enzyme normality was also titrated with TCI (Bender *et al.*, 1966) and was initially above 98% but dropped to 94–96% within 6 months' storage at 4°. Enzyme concentrations were determined by weight and corrected by the factors from the active site titrations. Stock solutions of CT (1 mg/ml of 10^{-3} M HCl–0.02 M $CaCl_2$) were stored for maximally 5 days at 4°; prior to assays they were appropriately diluted with the same solvent. Stock solutions of the subtilisins were prepared fresh daily at a concentration of 1–7 mg/ml of ice-cold 0.2 M acetate–0.02 M $CaCl_2$ (pH 6.0) (Glazer, 1967) and diluted with 0.02 M $CaCl_2$ to the appropriate concentrations immediately prior to a set of kinetic runs. Solutions containing less than 0.1 mg/ml of subtilisin were used within 3 hr since their activity dropped significantly upon prolonged storage.

Amino acids and derivatives were prepared according to reported procedures: Phe($2CH_3$)-HCl, Ala(1Nph)-HCl, Ala(2Nph)-HCl, Ac-Ala(2Nph), Ac-Ala(6Qui), and Ala(6Qui) (Berger *et al.*, 1973); Phe(4F)-HBr and Phe(F_5)-HBr (Bosshard and Berger, 1973); Phe(4I) (Abderhalden and Brossa, 1909); Phe($4NO_2$) (Bergel and Stock, 1954); Cys(Bzl)-HCl (Wood *et al.*, 1939); Cys(Dpm)-HCl (Hiskey and Adams, 1965); Tyr(Bzl)-OMe-HCl (Wünsch *et al.*, 1958); Tyr-OMe-HCl (Schwarz and Bumpus, 1959), Ac-Ala(1Nph), mp 178°, $[\alpha]_D^{24}$ 41.4° (*c* 0.5 in methanol–DMF, 1:2), was prepared by a method published previously (Berger *et al.*, 1973), Cys(Acm)-HCl (Veber *et al.*, 1972) was a gift from Dr. P. Bützer.

Other Reagents. Ac-Tyr-OEt and Ac-Ala-Ala-Ala-OMe were from Miles-Yeda, Rehovot, Israel. Succ-Phe-OMe was prepared by succinylating Phe-OMe-HCl (Boissonnas *et al.*, 1956) with succinic anhydride in aqueous solution at pH 9 (pH-Stat). TCI was synthesized according to Schonbaum *et al.* (1961) and recrystallized from cyclohexane before use, mp 131–133°; Schonbaum *et al.* (1961) report mp 133–133.5°.

Synthesis of inhibitors was accomplished by coupling the peptide succinimidooxy ester Z-Ala-Ala-ONSu (Bosshard *et al.*, 1973) with the free amino acids P_1 in DMF. Usually 2–5 mmol of the amino acid P_1 (occasionally in the form of the hydrochloride or hydrobromide) was dissolved or dispersed in 10–20 ml of DMF, and 2 equiv of *N*-methylmorpholine (3 equiv for the amino acid hydrochlorides or -bromides) was added. Z-Ala-Ala-ONSu (1 equiv) was added as a solid into the vigorously stirred solution, and the reaction was allowed to proceed for 3–5 hr at room temperature. The gel which formed normally upon neutralizing the amino acid salts with *N*-methylmorpholine dissolved within 5–10 min after addition of the active ester indicating the rapid progress of the coupling reaction. Peptides were precipitated from the reaction mixture with several volumes of 0.05 M HCl and either filtered off, washed (H_2O), and recrystallized or extracted into ethyl acetate. Organic extracts were washed (H_2O and saturated NaCl solution), dried ($MgSO_4$), and evaporated, and the products were recrystallized from appropriate solvents. Table I lists the peptides prepared, their analytical data, and the solvents used for recrystallization. Yields of pure peptides (homogeneous in various tlc systems and satisfactory elementary analysis) were between 60 and 80%, and in a few cases below 60%. Synthesis and analytical data of inhibitors which are not listed in Table I have been given elsewhere (Bosshard, 1973b).

Racemization during activation of Z-Ala-Ala to the suc-

[3] This nomenclature divides the enzyme's substrate binding site into subsites S_1, S_2 . . . from the site of the bond cleaved toward the amino end of the substrate. Accordingly, amino acid residues of the substrate or the inhibitor are designated P_1, P_2 . . . (Schechter and Berger, 1967).

TABLE I: Analytical Data of Peptides Z-Ala-Ala-P_1.

Residue P_1	Formula	Mp[a] (°C)	$[\alpha]_D^{20}$[b] (deg)	% Calcd C	% Calcd H	% Calcd N	% Found C	% Found H	% Found N
Ala	$C_{17}H_{23}N_3O_6$ (365.4)	221–222[k]	−10.4	55.80	6.35	11.50	55.92	6.30	11.62
Phe($2CH_3$)	$C_{24}H_{29}N_3O_6$ (455.5)	191–192[h]	−11.7	63.28	6.42	9.22	63.11	6.52	8.98
Phe(4F)	$C_{23}H_{26}FN_3O_6$ (459.5)[c]	160–162[j]	n.d.	60.12	5.70	9.15	59.91	5.63	9.51
Phe(F_5)	$C_{23}H_{22}F_5N_3O_6$ (531.4)[d]	208–209[m]	n.d.	51.98	4.17	7.91	52.28	4.22	8.15
Phe(4I)	$C_{23}H_{26}IN_3O_6$ (583.4)[e]	202–203[l]	6.2	47.35	4.84	9.6	47.42	4.93	9.82
Phe($4NO_2$)	$C_{23}H_{26}N_4O_8$ (486.5)	204[l]	−3.6	56.79	5.39	11.51	56.55	5.16	11.96
Tyr	$C_{23}H_{27}N_3O_7$ (457.5)	186–187[k]	9.0	60.39	5.95	9.19	60.30	5.99	9.25
Tyr(Bzl)	$C_{30}H_{33}N_3O_7$ (547.6)	170–171[i]	5.4	65.80	6.07	7.67	65.64	5.99	7.99
Ala(1Nph)	$C_{27}H_{29}N_3O_6$ (491.5)	209–210[i]	−18.1	65.98	5.95	8.55	65.72	5.89	8.35
Ala(2Nph)	$C_{27}H_{29}N_3O_6$ (491.5)	197–198[k]	5.8	65.98	5.95	8.55	65.78	6.03	8.41
Cys(Acm)	$C_{20}H_{28}N_4O_7$ (468.5)[f]	121–125[l]	−53	51.72	6.02	11.96	51.29	6.31	11.53
Cys(Bzl)	$C_{24}H_{29}N_3O_6S$ (487.6)[g]	169–170[i]	−30	59.12	5.96	8.62	59.30	5.70	8.83
Cys(Dpm)	$C_{30}H_{33}N_3O_6S$ (563.7)[h]	>87 dec[j]	−18.2	63.93	5.90	7.46	63.60	6.12	7.62

[a] Uncorrected. [b] Determined in DMF at a concentration of 10–20 mg/ml of solvent. [c] F calcd 4.13, found 3.92%. [d] F calcd 17.87, found 18.43%. [e] I calcd 21.75, found 21.65%. [f] S calcd 6.84, found 6.91%. [g] S calcd 6.58, found 6.32%; [h] S calcd 5.69, found 5.79%. [i] Recrystallized from water–ethanol. [j] Ethyl acetate–diethyl ether. [k] Ethyl acetate–diisopropyl ether. [l] Ethyl acetate–ethanol (*ca.* 10:1). [m] Precipitated from DMF with water.

cinimidooxy ester Z-Ala-Ala-ONSu and its subsequent coupling with a free amino acid has been studied by an enzymic technique for differentiating diastereomeric peptides (Bosshard *et al.*, 1973). The amount of configurational change was found to be not more than 2% in the most unfavorable case when the coupling was run in presence of 2 equiv of triethylamine. However, when up to 50% water was added to the reaction mixtures, the degree of racemization jumped to about 10%. Dry DMF was therefore used in all coupling reactions. Since the degree of overall racemization during the coupling step is dependent on the ratio of the velocity of the bimolecular coupling reaction to the velocity of the monomolecular racemization reaction of Z-Ala-Ala-ONSu, a fast coupling rate suppresses the configurational change. Tyr and Tyr(Bzl) are almost insoluble in DMF and thus react only very slowly with Z-Ala-Ala-ONSu. Therefore, the peptides Z-Ala-Ala-Tyr and Z-Ala-Ala-Tyr(Bzl) were prepared *via* the peptide methyl esters which were hydrolyzed subsequently to the peptide acids.

Details of the synthesis of Z-Ala-Ala-Ala(1Nph), Z-Ala-Ala-Tyr, and Z-Ala-Ala-Tyr(Bzl) are givn as illustrative examples.

Z-Ala-Ala-Ala(1Nph). Ala(1Nph)-HCl (0.8 g, 3.2 mmol) was dissolved in 15 ml of DMF. *N*-Methylmorpholine (1.07 ml, 9.6 mmol) and Z-Ala-Ala-ONSu (1.25 g, 2.3 mmol) were added into the vigorously stirred solution. The reaction mixture initially became jelly-like but cleared up within 5 min. After 2 hr at room temperature 150 ml of ice-cold 0.05 N HCl was added. The product separated out as an oil which was extracted into 3 × 100 ml of ethyl acetate. The organic extract was washed (2 × 100 ml of water and 100 ml of saturated NaCl solution), dried over $MgSO_4$, and evaporated *in vacuo* to leave a colorless oil which was crystallized from water–ethanol (1:1). The yield of pure peptide was 1.15 g (73%).

Z-Ala-Ala-Tyr. Tyr-OMe-HCl (463 mg, 2 mmol) was dissolved in 10 ml of DMF. *N*-Methylmorpholine (0.45 ml, 4 mmol) and Z-Ala-Ala-ONSu (784 mg, 2 mmol) were added, and the reaction mixture was stirred at ambient temperature for 5 hr. The solution was then poured into 200 ml of ice-cold 0.05 N HCl. The precipitated product was filtered off, washed with water, 5% $NaHCO_3$ solution, and again with water, and dried *in vacuo* over P_2O_5. The yield was 620 mg (64%) of colorless crystals, mp 157°, $[\alpha]_D^{18}$ −34° (*c* 1 in methanol).

Anal. Calcd for $C_{24}H_{29}N_3O_7$ (471.5): C, 60.67; H, 6.20; N, 8.91. Found: C, 60.48; H, 6.31; N, 8.93.

The above methyl ester (471 mg, 1 mmol) was suspended in 100 ml of 0.1 M KCl (1 mM in Na_2HPO_4). The pH was brought to 8.5 with 0.3 N KOH. Enzymic hydrolysis of the methyl ester was achieved by adding CT (4.2 mg). The pH was kept constant at 8.5 by means of a pH-Stat using 0.3 N KOH as titrant. More enzyme (4 mg each time) was added after 30 and 60 min. Base uptake stopped after 3 hr. The reaction mixture was diluted with 50 ml of saturated $NaHCO_3$ solution and filtered. The filtrate was extracted with 50 ml of chloroform to remove traces of unhydrolyzed peptide ester. The aqueous layer was acidified (0.5 N HCl) and extracted with 2 × 100 ml of ethyl acetate. The organic extract was dried ($MgSO_4$) and evaporated. The residue was once recrystallized from ethyl acetate–diisopropyl ether; yield 370 mg (81%).

Z-Ala-Ala-Tyr(Bzl). Tyr(Bzl)-OMe-HCl (644 mg, 2 mmol) was reacted with Z-Ala-Ala-ONSu exactly as outlined above for Z-Ala-Ala-Tyr-OMe. Z-Ala-Ala-Tyr(Bzl)-OMe was obtained as an oil which was homogeneous in thin-layer chromatography and which was used without further purification in the following step; 850 mg (*ca.* 1.5 mmol) of the oil was dissolved in 12 ml of ethanol and 3 ml of 1 M NaOH. Hydrolysis proceeded at 25° for 1.5 hr after which time the base strength of the reaction mixture remained constant indicating complete hydrolysis. The pH of the solution was brought to about pH 6 (1 N HCl), most of the ethanol evaporated, and the peptide acid precipitated by adding more 1 N HCl. The product was collected by filtration and recrystallized from ethyl acetate, yield 480 mg (58%).

Rate Measurements. Initial rates of enzymic hydrolysis of substrates were determined by the pH-Stat method (pH-Stat assembly by Radiometer, Copenhagen, composed of pH-meter 26, Titrator 11, Autoburette ABU 12, Titration As-

TABLE II: Experimental Conditions for Enzymic Reactions and $\bar{K}_m$(app) Values Used in Calculating $\bar{K}_i$ According to Equation 1.[a]

Enzyme	$[E_0]$[b] (M)	Substrate	$[S_0]$[b] (mM)	$\bar{K}_m$(app)[c] (M^{-1})
CT	1.1×10^{-8}	Ac-Tyr-OEt	0.25–0.4	
	(1.6×10^{-8})	Ac-Tyr-OEt	(0.35)	1200
	1.2×10^{-7}	Succ-Phe-OMe	1–15	
	(5.5×10^{-7})	Succ-Phe-OMe	(1)	310
S. Novo	1.2×10^{-7}	Ac-Tyr-OEt	1–10	
	(2.0×10^{-7})	Ac-Tyr-OEt	(1.5)	15
	2.1×10^{-9}	Ac-(L-Ala)$_3$-OMe	0.4–6.1	
	(5.3×10^{-9})	Ac-(L-Ala)$_3$-OMe	(0.4)	620
	6.0×10^{-7}	TCI	0.008–0.125	
S. Carlsberg	2.9×10^{-8}	Ac-Tyr-OEt	0.75–10	
	(4.1×10^{-8})	Ac-Tyr-OEt	(1.5)	55
	0.9×10^{-9}	Ac-(L-Ala)$_3$-OMe	0.4–6.7	
	(3.1×10^{-9})	Ac-(L-Ala)$_3$-OMe	(0.4)	530
	2.5×10^{-7}	TCI	0.008–0.125	

[a] pH 6.5, 25 ± 0.2°, 0.15 M KCl. [b] Concentrations in parentheses are for experiments performed to determine $\bar{K}_i$ values according to eq 1 (fixed [S] and varying [I]). [I] was varied such as to give 6–10 values for v/v_i between about 1.2 and 4 (see Figure 1A). The initial velocity v in absence of inhibitor was determined by taking the average of at least five rate determinations. [c] Mean values of 4–5 individual determinations.

sembly TTA 31 and Servograph REC 51). Reactions were run in a total volume of 2.00 ml at 25.0 ± 0.2° (water-jacketed cell) and at an ionic strength of 0.15 (KCl). The titrant was 0.005 N NaOH. Dry argon was blown over the surface of the reaction mixture to exclude CO_2. Reactions were initiated by adding the enzyme in a volume of 10–20 μl. In every case, the initial portion of alkali uptake *vs.* time plots was linear, and initial rates were determined directly from the slopes. Less than 10% of substrate was hydrolyzed during the time necessary to evaluate initial velocities.

Concentrations of enzymes and substrates used in the rate measurements are given in Table II. Stock solutions of substrates in 0.15 M KCl were 0.01 M for Ac-Tyr-OEt and 0.02 M for Ac-Ala-Ala-Ala-OMe and Succ-Phe-OMe. Stock solutions of inhibitors were prepared by dissolving the inhibitors in 0.15 M KCl by the aid of a few drops of 1 N KOH; immediately afterwards the pH was adjusted to about 6.5 with HCl. Concentrations were 0.01–0.001 M.

Hydrolysis of TCI by subtilisins at 25° and pH 7.0 (0.04 M phosphate buffer, ionic strength adjusted to 0.15 with KCl, 3.3% (v/v) acetonitrile) was monitored at 335 nm on a Gilford 2400-S spectrophotometer according to "method A" of Bender *et al.* (1966). An extinction coefficient of 9.04×10^3 M^{-1} cm^{-1} at 335 nm was used for TCI (Bender *et al.*, 1966). TCI stock solution was 4×10^{-3} M in dry acetonitrile and was prepared fresh daily. Assay volumes of totally 3.00 ml were composed of substrate (in 5–100 μl of acetonitrile), inhibitor (in phosphate buffer), buffer, and enough acetonitrile to give a final concentration of 3.3% (v/v). Reactions were started by adding the enzyme in a volume of 10 μl. For concentration ranges of substrate and enzyme see Table II. Initial velocities were measured as in the pH-Stat assay.

Fluorescence Measurements. Fluorescence quenching of the naphthyl group in presence of increasing amounts of CT was determined on the instrument described by Teichberg and Shinitzky (1973).

Analysis of Reaction Rate Data. In all experiments the total enzyme concentration $[E_0]$ was always well below the concentration of substrates [S] and inhibitors [I]. In the following equations [S] and [I] are therefore taken as their total concentrations $[S_0]$ and $[I_0]$. Initial velocities from reactions at varying [S] and in absence or in presence of fixed [I] were plotted in the usual double reciprocal manner.

The ratio of the reaction rates in absence (v) and in presence (v_i) of a competitive inhibitor is given by

$$v/v_i = 1 + \bar{K}_i[I]/(1 + \bar{K}_m(app)[S]) \quad (1)$$

Initial rates from experiments at fixed [S] and varying [I] were plotted according to eq 1 (Figure 1A), and $\bar{K}_i$ was determined from the slope using $\bar{K}_m$(app) (Table II) obtained from the double reciprocal plot. In order to test the stoichiometry of inhibitor binding, the same data were plotted (Figure 1B) according to

$$\log (v/v_i - 1) = \{\log \bar{K}_i/(1 + \bar{K}_m(app)[S])\} + \log [I] \quad (2)$$

Inhibition of TCI hydrolysis by S. Novo and S. Carlsberg was noncompetitive in the general sense that substrate and inhibitor did not compete for the same site, and thus a ternary enzyme–substrate–inhibitor complex could be formed (Bosshard, 1973a). The simplest model accounting for all interactions detected is given by

$$\begin{array}{ccccc} E & \overset{\bar{K}}{\rightleftharpoons} & ES & \overset{k}{\longrightarrow} & E + P \\ {\scriptstyle \bar{K}_i}\downarrow\uparrow & & \downarrow\uparrow{\scriptstyle \alpha\bar{K}_i} & & \\ EI & \rightleftharpoons & ESI & & \\ & {\scriptstyle \alpha\bar{K}} & & & \end{array} \quad (3)$$

ES stands for the enzyme–substrate complex, EI for the enzyme–inhibitor complex, ESI for the ternary complex, and P for products. k is the rate of product formation from ES, and α is a proportionality factor. Equilibrium between all complexes with the enzyme is assumed; *i.e.*, the rate of breakdown of ES into products is small enough not to disturb the equilibrium (Bender *et al.*, 1966). Then, the following equilibrium equations can be formulated (eq 4).

$$[ES] = \bar{K}[E][S], [EI] = \bar{K}_i[E][I], [ESI] = \alpha\bar{K}\bar{K}_i[E][S][I] \quad (4)$$

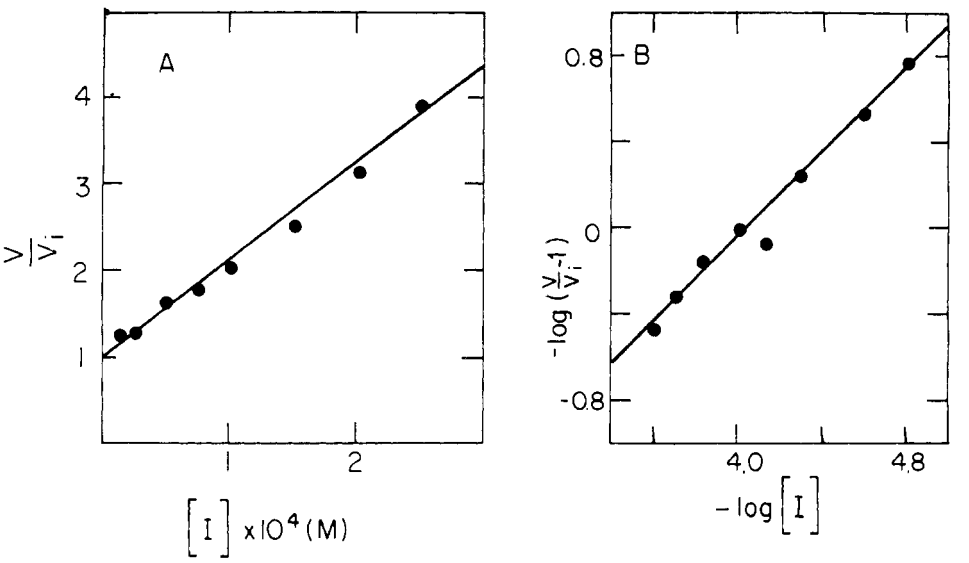

FIGURE 1: Inhibition of the CT catalyzed hydrolysis of Ac-Tyr-OEt by Z-Ala-Ala-Ala(1Nph): (A) data plotted according to eq 1; (B) same data plotted according to eq 2. The slope in B is 0.98.

From (4) and

$$[E_0] = [E] + [ES] + [EI] + [ESI] \quad (5)$$

it follows that

$$[ES] = \bar{K}[E_0][S]/(1 + \bar{K}[S] + \bar{K}_i[I] + \alpha\bar{K}\bar{K}_i[S][I]) \quad (6)$$

The reciprocal reaction rate $1/v_i$ in presence of inhibitor is therefore

$$1/v_i = 1/k[ES] = (1 + \bar{K}_i[I])/k\bar{K}[E_0][S] + (1 + \alpha\bar{K}_i[I])/k[E_0] \quad (7)$$

TABLE III: Inhibition Constants, $\bar{K}_i$ ($=1/K_i$), and Standard Free Energies of Binding, $\Delta F°$, of Tripeptides Z-Ala-Ala-P_1 and N^α-Acetylamino Acids toward CT.[a]

Inhibitor Residue P_1	$\bar{K}_i$ (M^{-1})	$-\Delta F°$ (kcal/mol)	Inhibitor Residue P_1	$\bar{K}_i$ (M^{-1})	$-\Delta F°$ (kcal/mol)
Cys(Acm)	1,400	4.3	Phe(4F)	12,000	5.6
Ala(6Qui)	2,600	4.7	Phe($4CH_3$)	13,000	5.6
Ala[b]	2,900	4.7	Ala(1Nph)	16,000	5.7
Phe(F_5)	3,100	4.8	Cys(Dpm)	23,000	6.0
Leu	3,100	4.8	Ala(2Nph)	28,000	6.1
Phe($2CH_3$)	3,300	4.8	Trp	36,000	6.2
Phe	4,500	5.0	Phe($4NO_2$)	51,000	6.4
Tyr	8,400	5.4			
Phe(3F)[b]	9,000	5.4	Ac-Ala(6Qui)	100	2.7
Cys(Bzl)	9,200	5.4	Ac-Ala(1Nph)	330	3.4
Tyr(Bzl)	9,800	5.4	Ac-Trp[c]	680	3.9
Phe(41)	12,000	5.6	Ac-Ala(2Nph)	1,000	4.1

[a] Inhibition determined at pH 6.5 by assaying the esterase activity toward Ac-Tyr-OEt and Succ-Phe-OMe. Further details are given in Table II. The values are means of three independent measurements, two of which were against Ac-Tyr-OEt. [b] Mean of only two measurements against Ac-Tyr-OEt. [c] Determined by equilibrium dialysis at pH 6.6 in 0.1 M phosphate at 5° (Johnson and Knowles, 1966).

Equation 7 reduces to the case of competitive inhibition for $\alpha = 0$ and of "simple noncompetitive" inhibition (Laidler, 1958) for $\alpha = 1$. Equation 7 can be extended and rearranged to

$$(v/v_i)[I]/(1 - v_i/v) = 1/(\bar{K}_i + \alpha\bar{K}\bar{K}_i[S]) + \bar{K}[S]/(\bar{K}_i + \alpha\bar{K}\bar{K}_i[S]) \quad (8)$$

For competitive and simple noncompetitive inhibition eq 8 gives straight lines with slopes of $\bar{K}/\bar{K}_i$ and 0, respectively. For $0 < \alpha < 1$ and for $\alpha > 1$ eq 8 gives rise to curves which are bent downwards or upwards, respectively. For infinite [S] the right-hand portion of eq 8 reduces to $1/\alpha\bar{K}_i$ and for [S] = 0 to $1/\bar{K}_i$. Data from the inhibition of TCI hydrolysis were plotted in the double reciprocal mode and according to eq 8 (Figures 2 and 3). $\bar{K}_i$ and α were roughly estimated from the intercepts and from extrapolation to infinite [S].[4]

Straight lines in double reciprocal plots and in graphs according to eq 1 and 2 were fitted by linear least-squares analysis of data points (Hewlett-Packard Calculator 9820 A). On the basis of duplicate to quadruplicate measurements of the inhibition constants toward different substrates, $\bar{K}_i$ values reported in Tables III and IV are estimated to be reproducible within ±20% or better.

Results

Type of Reversible Inhibition by Z-Ala-Ala-P_1. The inhibition of the CT catalyzed hydrolysis of Ac-Tyr-OEt and Succ-Phe-OMe was of the competitive type for all inhibitors tested. Inhibition of S. Novo and S. Carlsberg determined against the substrates Ac-Ala-Ala-Ala-OMe and Ac-Tyr-OEt was also exclusively competitive by all tripeptide inhibitors. But the same peptides inhibited TCI hydrolysis by the subtilisins noncompetitively. The inhibition was of the simple noncompetitive type (Laidler, 1958; $\alpha = 1$ in eq 7) with Z-Ala-Ala-Phe and Z-Ala-Ala-Tyr. Z-Ala-Ala-Cys(Bzl) and Z-Ala-Ala-Cys(Dpm) showed a more complex inhibition pattern

[4] An accurate determination of $\bar{K}_i$ and α would need more double reciprocal plots at different [I] and replotting slopes and intercepts according to eq 7 against varying [I].

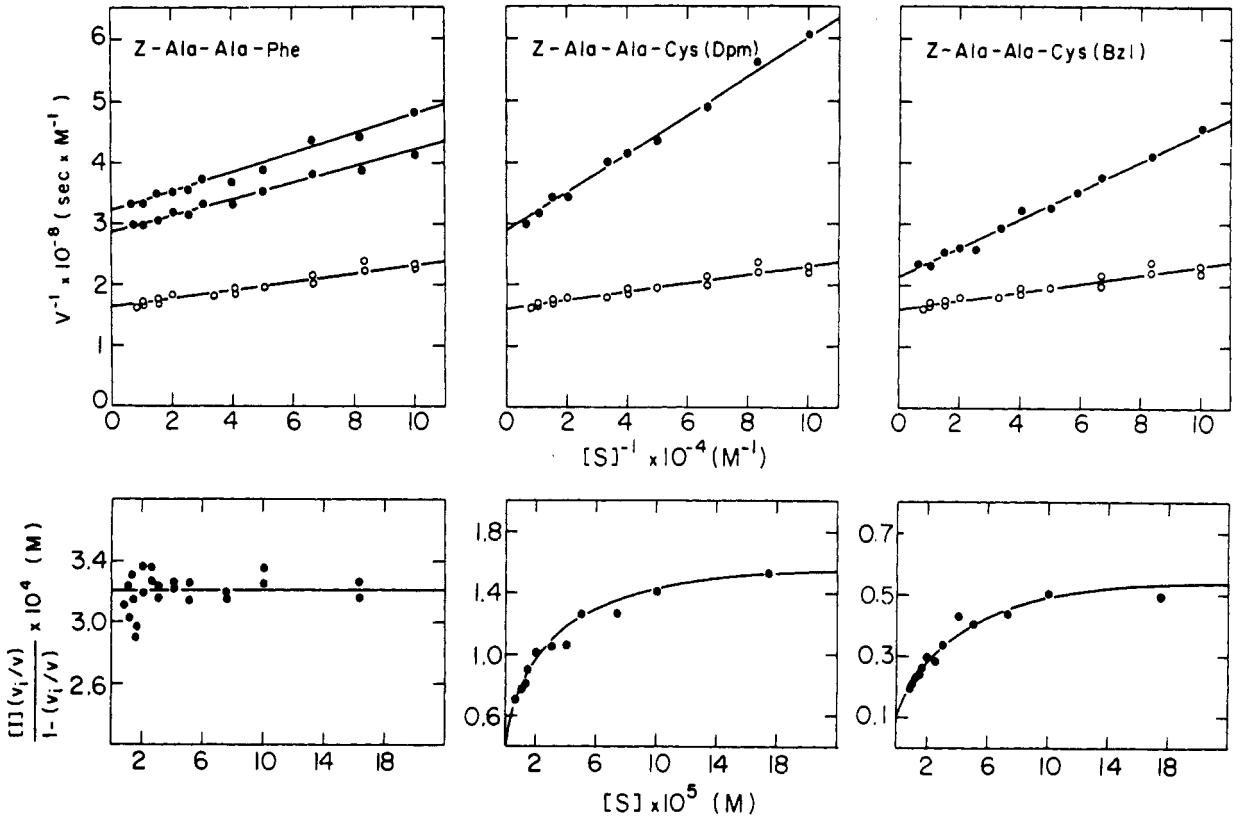

FIGURE 2: Inhibition of the S. Novo catalyzed hydrolysis of TCI by Z-Ala-Ala-Phe (2.5×10^{-4} and 3.25×10^{-4} M), Z-Ala-Ala-Cys(Dpm) (1.25×10^{-4} M), and Z-Ala-Ala-Cys(Bzl) (2×10^{-5} M); (○) reaction in absence of inhibitor. The lower panels show the data of the double reciprocal plots redrawn according to eq 8. Estimated values for $\bar{K}_i$ and α: Z-Ala-Ala-Phe, $\bar{K}_i = 3000\ M^{-1}$, $\alpha = 1$; Z-Ala-Ala-Cys(Dpm), $\bar{K}_i = 20{,}000\ M^{-1}$, $\alpha = 0.3$; Z-Ala-Ala-Cys(Bzl), $\bar{K}_i = 100{,}000\ M^{-1}$, $\alpha = 0.2$.

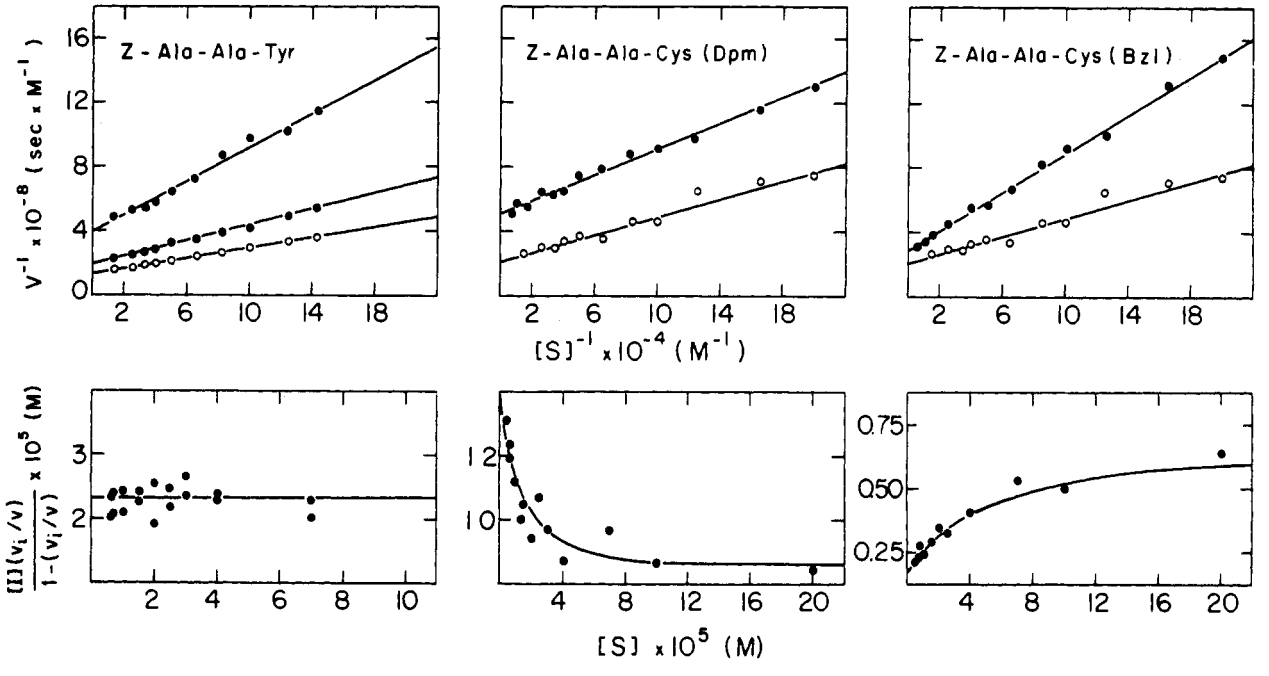

FIGURE 3: Inhibition of the S. Carlsberg catalyzed hydrolysis of TCI by Z-Ala-Ala-Tyr (10^{-5} and 5×10^{-5} M), Z-Ala-Ala-Cys(Dpm) (10^{-4} M), and Z-Ala-Ala-Cys(Bzl) (2×10^{-6} M); (○) reaction in absence of inhibitor. The lower panels show the data of the double reciprocal plots redrawn according to eq 8. Estimated values for $\bar{K}_i$ and α: Z-Ala-Ala-Tyr, $\bar{K}_i = 43{,}000\ M^{-1}$, $\alpha = 1$; Z-Ala-Ala-Cys(Dpm), $\bar{K}_i = 7000\ M^{-1}$, $\alpha = 2$; Z-Ala-Ala-Cys(Bzl), $\bar{K}_i = 500{,}000\ M^{-1}$, $\alpha = 0.33$.

characterized by α values of around 0.2–0.3 and of 2 in the case of the interaction of Z-Ala-Ala-Cys(Dpm) with S. Carlsberg (Figures 2 and 3). In contrast, Bz-Arg inhibited competitively the TCI hydrolysis by S. Novo and S. Carlsberg (Wright, 1972; Bosshard, 1973a) but noncompetitively the hydrolysis of peptide esters such as Ac-Ala-Ala-Ala-OMe. This inhibition pattern by Bz-Arg demonstrated (Bosshard, 1973a) the binding of Ac-Ala-Ala-Ala-OMe to the peptide binding

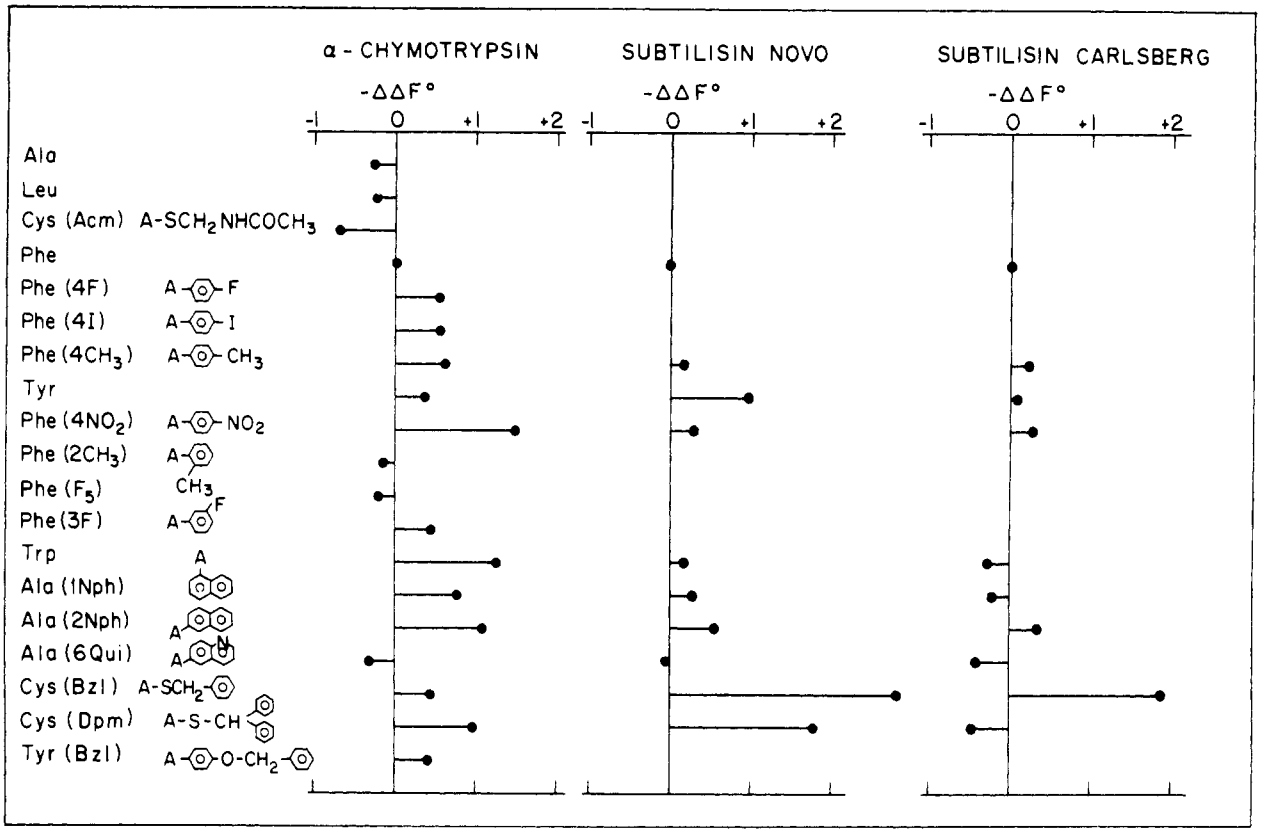

FIGURE 4: Differences in standard free energies of binding ($\Delta\Delta F°$ in kcal/mol) between Z-Ala-Ala-P_1 and Z-Ala-Ala-Phe bound to CT, S. Novo, or S. Carlsberg. Data are taken from Tables III and IV. Amino acids P_1 and structural formulas of some side chains (A = CH_2) are given in the first column.

site which is the A site according to the nomenclature of Wright (1972).

Enzymic hydrolysis of inhibitors was checked by incubating the inhibitors (10^{-2} M) for 1 hr at pH 7.0 (0.1 M Tris-HCl) and 25° in presence of *ca.* 10^{-6} M CT, S. Novo, or S. Carlsberg. Reaction mixtures were analyzed by thin-layer chromatography (silica gel plates, upper phase of a mixture of 1-butanol acetic acid–water, 25:6:25, as mobile phase). No hydrolysis occurred as indicated from the absence of any ninhydrin positive amino acids or dipeptides and from the presence of only unchanged starting material.

TABLE IV: Inhibition Constants, $\bar{K}_i$ ($=1/K_i$), and Standard Free Energies of Binding, $\Delta F°$, for Tripeptides Z-Ala-Ala-P_1 toward S. Novo and S. Carlsberg.[a]

	S. Novo		S. Carlsberg	
Residue P_1	$\bar{K}_i$ (M^{-1})	$-\Delta F°$ (kcal/mol)	$\bar{K}_i$ (M^{-1})	$-\Delta F°$ (kcal/mol)
Ala(6Qui)	3,300	4.8	15,000	5.7
Phe	3,600	4.9	30,000	6.1
Phe($4CH_3$)	4,500	5.0	42,000	6.3
Trp	4,600	5.0	19,000	5.8
Ala(1Nph)	5,500	5.1	20,000	5.9
Phe($4NO_2$)	5,800	5.1	44,000	6.3
Ala(2Nph)	9,200	5.4	51,000	6.4
Tyr	17,000	5.8	35,000	6.2
Cys(Dpm)	71,000	6.6	13,000	5.6
Cys(Bzl)	280,000	7.4	700,000	8.0

[a] Inhibition at pH 6.5 determined by assaying the esterase activity toward Ac-Ala_3-OMe and Ac-Tyr-OEt. For experimental details see Table II. The values are averages of two determinations.

Inhibition Constants. $\bar{K}_i$ values for reversible, competitive inhibition were obtained graphically from plots of v/v_i *vs.* [I] at constant [S] (eq 1, Figure 1A). Values for $\bar{K}_i$ and $\Delta F°$ ($= -RT \ln \bar{K}_i$) are compiled in Tables III and IV. Estimated values[4] for $\bar{K}_i$ and α for the noncompetitive inhibition of TCI hydrolysis by the subtilisins are given in the explanations to Figures 2 and 3. The kinetically deduced binding constant for Z-Ala-Ala-Ala(1Nph) to CT at pH 7.0 was checked independently by the method of proflavine displacement (Bernhard *et al.*, 1966) and by fluorescence titration. The three values are in good agreement (Table V).

$\Delta F°$ values of Tables III and IV were standardized on Z-Ala-Ala-Phe in order to compare the inhibition of the three enzymes. Differences in standard free energies of binding, $\Delta\Delta F°$, between Z-Ala-Ala-P_1 and Z-Ala-Ala-Phe, are displayed in Figure 4.

pH Dependence of Inhibition Constants. Since $\bar{K}_m$(app) for aromatic acylamino acid esters is virtually independent of pH in the range from about pH 6 to 9 (Bender *et al.*, 1964; Glazer, 1968), the percentage of inhibition reflects the variation of $\bar{K}_i$ with pH. Per cent inhibition at fixed substrate, enzyme, and inhibitor concentration was determined over this pH-range. A pK_a of 7.1 was found to govern the inhibition of CT by Z-Ala-Ala-Ala(1Nph), Z-Ala-Ala-Cys(Dpm), Z-Ala-Ala-Tyr(Bzl), and Z-Ala-Ala-Ala (Figure 5). S. Novo and S. Carlsberg showed pK_a values of 7.3 and 7.4, respectively, with Z-Ala-Ala-Tyr and Z-Ala-Ala-Cys(Bzl) (Figures 6 and 7). Inhibition constants were therefore determined at pH 6.5

TABLE V: Binding of Z-Ala-Ala-Ala(1Nph) to CT. Comparison of Binding Constants, $\bar{K}_b$, Determined by Different Methods.[a]

Method	$\bar{K}_b$ (M^{-1})
Inhibition of substrate hydrolysis (pH-Stat)	7800 ± 950 (s.d.m.)[d]
Proflavine displacement[b,c]	6500 ± 1100 (s.d.m.)[d]
Fluorescence titration[c]	~8000

[a] pH 7.0 (0.05 M phosphate except in pH-Stat experiments), 25°, ionic strength adjusted to 0.15 with KCl. [b] According to Bernhard *et al.* (1966). Dye was 0.94×10^{-5} M, CT 0.91×10^{-4} M, and inhibitor from 3×10^{-5} to 1.4×10^{-3} M. K_b for proflavine was determined independently and was 2.2×10^4 M^{-1}. $\Delta\epsilon^{465} = 1.85 \times 10^4$ M^{-1} cm^{-1} for the CT–proflavine complex was taken from Bernhard *et al.* (1966). [c] In calculating K_b no correction was made for possible enzyme oligomerization which could have reduced the concentration of inhibitor binding monomeric enzyme (Faller and LaFond, 1971). However, at pH 7.0 and $[E_0]$ from 10^{-5} to 5×10^{-4} M monomeric enzyme predominates (Aune and Timasheff, 1971; Faller and LaFond, 1971). [d] Means of four and five determinations, respectively.

where they depend only slightly on pH and have almost maximal absolute values.

Stoichiometry of Inhibitor Binding. One inhibitor molecule was found per active site of all three enzymes as deduced from a slope of 1 of plots according to eq. 2. Figure 1B gives an example.

Discussion

One important assumption in mapping a binding site by comparing binding constants of ligands is a uniform binding mode of all ligands to a single macromolecular binding site. A similar assumption holds of course for the comparison of catalytic constants of substrates. However, in most cases uniformity of the binding mode is hard to prove rigorously, and this crucial point is often passed by silently. In our case too, we shall only be able to strengthen but not prove this assumption.

The possibility of different ways of binding of a ligand to a binding site diminishes when the number of topographically fixed interactions, say hydrogen bonds, salt bridges, van der Waals interactions, between ligand and binding site increases. Whence, since our aim was to compare the binding of various amino acid side chains to the specificity determining subsites S_1, we forced the P_1 residues into entering the S_1 loci by adding additional invariable points of contact. This was achieved by combining the variable P_1 residue with the constant part Z-Ala-Ala. Crystallographic work showed in the meantime that this was a good choice. Peptide chloromethyl ketones bound to γ-chymotrypsin revealed interactions between subsites S_1–S_3 and residues P_1–P_3 (Segal *et al.*, 1971). The interactions are predominantly with an extended segment of the main chain of the enzyme (Ser-214 to Gly-216) which forms an antiparallel β structure. Hydrogen bonds are formed between C=O of Ser-214 (S_1) and NH of P_1 and between NH and CO of Gly-216(S_3) and C=O and NH of P_3. All these contacts are in addition to the binding of the variable residue P_1 to the hydrophobic pocket in S_1

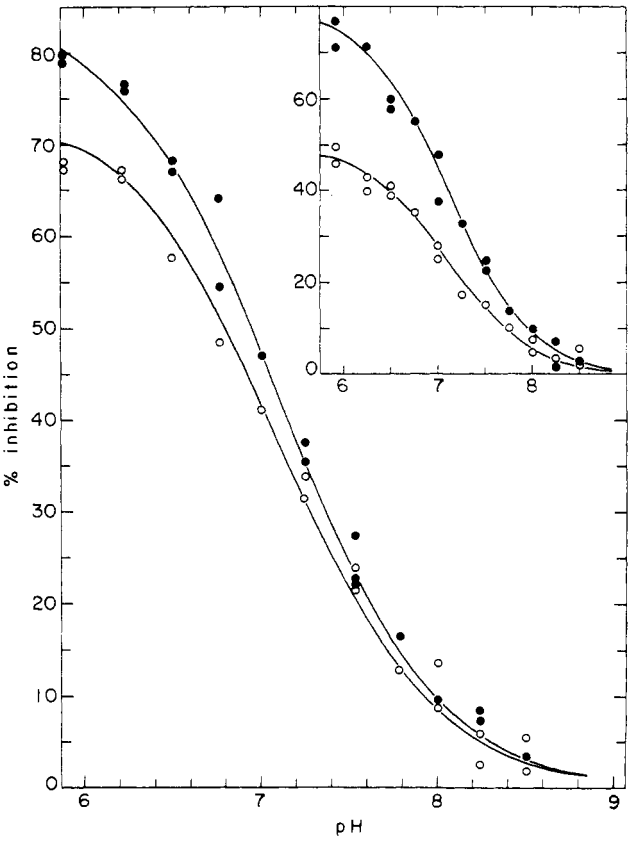

FIGURE 5: pH dependence of the inhibition of CT by tripeptides. Inhibition determined by assaying the esterase activity toward 3.5×10^{-4} M Ac-Tyr-OEt in 0.15 M KCl at 25° at an enzyme concentration of 2×10^{-8} M in absence and in presence of 1.5×10^{-4} M Z-Ala-Ala-Ala(1Nph) (●) and 10^{-4} M Z-Ala-Ala-Cys(Dpm) (○). Inset: inhibition by 2.5×10^{-4} M Z-Ala-Ala-Tyr(Bzl) (○) and 1.25×10^{-3} M Z-Ala-Ala-Ala (●). The curves drawn are calculated for a single protonatable group of $pK_a = 7.1$.

(Steitz *et al.*, 1969). Analogous X-ray work has been reported with S. Novo. Peptide chloromethyl ketones (Robertus *et al.*, 1972a) and inhibitors (virtual substrates, Robertus *et al.*, 1972b) of similar structures as those reported here have been studied. Again, there is an antiparallel β structure (Ser-125 to Gly-127) on the enzyme which, in case of the inhibitors, provides two hydrogen bonds for the S_3–P_3 interaction. Surprisingly, there is a hydrophobic S_4–P_4 contact with inhibitors such as Z-Gly-Gly-Tyr where the phenyl ring of the benzyloxycarbonyl group binds to the well-defined S_4 site. S_4 seems to have a high affinity for aromatic side chains since the dipeptide Z-Ala-Phe is bound in a "reverse" mode with the P_1 residue Phe interacting with S_4. In all our inhibitors we offer the subsite S_4 of S. Novo a benzyloxycarbonyl group. In the crystalline S. Novo–inhibitor complex the S_1–P_1 interaction is not uniform for all inhibitors. While, as expected for an aromatic binding site, Phe and Tyr bind to the S_1 crevice, Leu and Arg show two binding modes, one with their side chains in the S_1 crevice, the other with the side chains twisted away near to His-64. These observations weaken the above assumption of a uniform binding mode of our inhibitors in S_1 of the subtilisins.

The pH dependence of inhibitor binding provides another indication for uniqueness of binding. Acetylamino acids, which in the CT crystal are bound with the carboxyl group near to His-57 and Ser-195 of the catalytic locus (Steitz *et al.*, 1969), inhibit CT with $\bar{K}_i$ increasing with decreasing pH. A pK_a of 7.3 was assigned to a single protonatable group

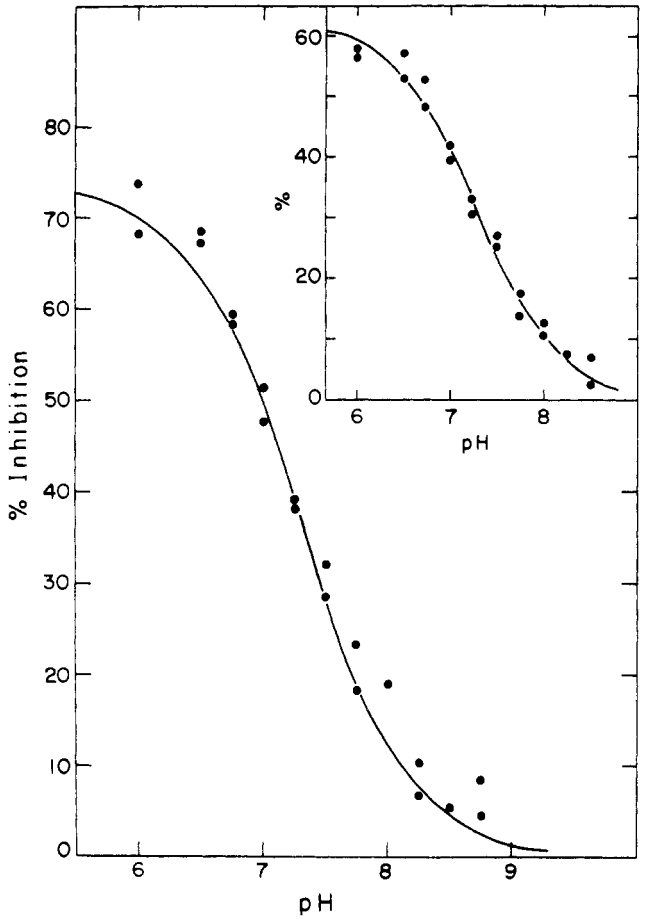

FIGURE 6: pH dependence of the inhibition of S. Novo by 10^{-5} M Z-Ala-Ala-Cys(Bzl). Inhibition was determined by assaying the esterase activity toward 1.25×10^{-3} M Ac-Tyr-OEt in 0.15 M KCl at 25° and at an enzyme concentration of 6.5×10^{-8} M in absence and in presence of inhibitor. Inset: inhibition by 10^{-4} M Z-Ala-Ala-Tyr. The curves drawn are calculated for a single protonatable group of $pK_a = 7.3$.

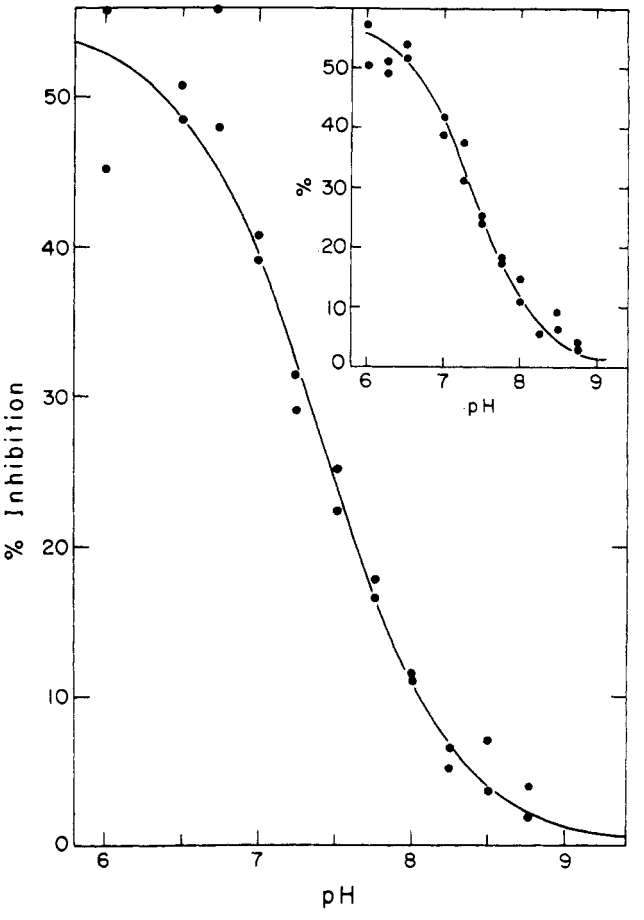

FIGURE 7: pH dependence of the inhibition of S. Carsberg by 1.25×10^{-6} M Z-Ala-Ala-Cys(Bzl). Inhibition was determined by assaying the esterase activity toward 1.5×10^{-3} M Ac-Tyr-OEt in 0.15 M KCl at 25° at an enzyme concentration of 1.2×10^{-8} M in absence and in presence of inhibitor. Inset: inhibition by 4×10^{-5} M Z-Ala-Ala-Tyr. The curves drawn are calculated for a single protonatable group of $pK_a = 7.4$.

(Johnson and Knowles, 1966). The net negative charge of active site Asp-102 was held responsible for the repelling of the carboxyl group of acetylamino acids (Johnson and Knowles, 1966; Gammon *et al.*, 1972). This negative charge is neutralized by the protonated His-57. The pK_a governing inhibitor binding was therefore assigned to the active site His-57. Assuming an inhibitor would bind in a "reverse" way (see above for Z-Ala-Phe bound to S. Novo), its carboxyl group could no longer feel the negative charge of Asp-102, or at least the absolute values of $\bar{K}_i$ would depend little on pH (see Dixon, 1953, for a quantitative treatment). A single protonatable group of $pK_a = 7.1$ was found responsible for the inhibition of CT by Z-Ala-Ala-Ala(1Nph), Z-Ala-Ala-Cys(Dpm), Z-Ala-Ala-Tyr(Bzl), and Z-Ala-Ala-Ala (Figure 5). Four widely different P_1 residues were chosen in purpose, with only Ala(1Nph) being a typical "CT-substrate" (Hayashi and Lawson, 1969). The equal dependence on a single pK_a implies that at least a grossly differing binding mode as, *e.g.*, "reverse" binding, can be excluded for any one of the four inhibitors tested and highly probably also for all other tripeptides listed in Table III.

Binding of inhibitors to the subtilisins is also dependent on a single pK_a of 7.3 for S. Novo (Figure 6) and 7.4 for S. Carlsberg (Figure 7). The latter value is in good agreement with a pK_a of 7.6 attributed to His-64 from fluorescence titration of the single Trp-113 in S. Carlsberg (R. S. Roche and M. Shinitzky, personal communication).

Still another problem remains, namely the role of the inhibitors as virtual substrates. In other words, are the kinetically determined $\bar{K}_i$ values real binding constants of the equation $E + I \rightleftarrows EI$ or rather overall equilibrium constants of the two-step process $E + I \rightleftarrows EI \rightleftarrows EA$, where EA is the enzyme acylated by the inhibitor? ^{18}O-Exchange between *N*-acetyl-L-amino acids (but not the D isomers) and water in presence of CT could have indicated enzyme acylation (Bender and Kemp, 1957). Significant EA formation is to be expected only around the pH optimum of the enzymic reaction which is about 8 for CT and above 8 for the subtilisins. The effect would be to increase $\bar{K}_i$ around these pH values. No such observation was made (Figures 5–7). Moreover, the tabulated values of $\bar{K}_i$ are from experiments at pH 6.5 where acylation is much less likely.

Having established good reasons for a uniform binding mode of all inhibitors and evidence that the kinetically measured $\bar{K}_i$'s are real binding constants, we shall now discuss the results of Tables III and IV and Figure 4. Clearly, CT shows the expected affinity for aromatic side chains in S_1. The hydrophilic acetamidomethyl group in Cys(Acm) (Z-Ala-Ala-Cys(Acm) is water soluble in contrast to all other inhibitors), the methyl side chain of Ala, and the hydrophobic but nonaromatic hydrocarbon portion of Leu all bind poorly to S_1

compared with Tyr or Trp. Two factors increase binding: substitution in the para and, probably, meta position of the phenyl ring and enlargement of the side chain from phenyl to naphthalene or indole. A $\Delta F°$ of -0.4 to -0.6 kcal/mol is gained from Phe to Tyr, Phe(3F), Phe(4F), Phe(4I), and Phe($4CH_3$). A similar increase in binding energy occurs when the phenyl ring is moved away from C_α of P_1, *i.e.*, probably pushed deeper into the S_1 pocket, by insertion of $-SCH_2-$ in Cys(Bzl). The largest increase in binding energy is found with Phe($4NO_2$). This is tentatively explained by the pronounced hydrophobicity of the *p*-nitrophenyl group and by a possible hydrogen bond between the nitro group and Ser-189 which lies deep in the hydrophobic pocket (Steitz *et al.*, 1969). Such a hydrogen bond might be formed also with Tyr, Phe(4I), and Phe(4F) or Phe(3F) and could account for the increased binding upon substituting the para position. The high $\bar{K}_i$ of Phe($4CH_3$) has to be explained by hydrophobic interaction alone. Improved binding of Trp, Ala(2Nph), and Ala(1Nph), the last amino acid being geometrically more related to Trp than Ala(2Nph) (Hayashi and Lawson, 1969), compares well with an earlier hypothesis of Niemann (Wallace *et al.*, 1963), that the S_1 site "has greater length than breadth" and "is not straight but curved." Slightly decreased binding of Phe(F_5) and Phe($2CH_3$) can be accounted for by hindered rotation around the C_β–C_γ bond, precluding optimal orientation of the phenyl ring in S_1.

Exceptional and striking are the high $\bar{K}_i$ values for Z-Ala-Ala-Tyr(Bzl) and Z-Ala-Ala-Cys(Dpm) as well as the loss in $\Delta F°$ of 1.4 kcal/mol from Ala(2Nph) to Ala(6Qui). Ala(2Nph) and Ala(6Qui) are geometrically isosteric (see Figure 4 for structural formulas). The first two observations could indicate that the S_1 pocket is much longer [Tyr(Bzl)] and broader [Cys(Dpm)] than hitherto anticipated. This would, however, conflict with the data from X-ray analysis (Steitz *et al.*, 1969). The pH dependence of the inhibition (Figure 5) has to be interpreted by an electrostatic interaction, similar to that observed for Z-Ala-Ala-Ala(1Nph) whose P_1 side chain is certainly well adapted by the S_1 pocket. Direct electrostatic interactions are long-range forces (Hirschfelder, 1965). The same pH dependence would therefore also be observed with

$$\underset{\alpha}{\overset{|}{-CH}}-COO^-$$

of P_1 tilted away by a few Å from the catalytic site in order to accomodate the aromatic side chain (or part of it) to the hydrophobic S_1 pocket. Such an interpretation would stress the high affinity of S_1 for aromatic structures even when binding has to be paid for by an improper fit of the residual part of the inhibitor to the extended binding site. The predilection for hydrophobic side chains in P_1 also explains the decreased binding of Ala(6Qui), the aromatic nitrogen of which is hydrated in the aqueous medium but probably not so when bound to the S_1 pocket. The pK_a of the quinoline nitrogen in Z-Ala-Ala-Ala(6Qui) is 4.9 as determined spectrophotometrically and potentiometrically (unpublished experiments). A protonated quinoline ring can therefore not be responsible for the decreased binding at pH 6.5.

The gain in binding energy due to the constant part of the inhibitors is 2 kcal/mol or more. The value originates from the difference in $\Delta F°$ between the acetylamino acids given at the end of Table III and the corresponding tripeptide inhibitors.

Subsites S_1 of S. Novo and S. Carlsberg respond little to changes in the structure of P_1 (Table IV). Differences in $\Delta F°$ values for Phe, Phe($4CH_3$), Phe($4NO_2$), Trp, and Ala(1Nph) are within about 0.2 kcal/mol for S. Novo which is near to the experimental error. The same differences are within 0.5 kcal/mol for S. Carlsberg. S. Carlsberg shows a markedly higher affinity for most of the inhibitors. $\Delta F°$ values are higher by 0.8–1.2 kcal/mol for all but two inhibitors. We attribute this to a more hydrophobic subsite S_1 with a strong S_1–P_1 interaction rather than to increased binding of the constant part of the inhibitors to S_2 to S_4. Barel and Glazer (1968) observed decreased K_m(app) and increased V_{max} values with S. Carlsberg for a series of acetylamino acid esters. They suggested on these grounds a less polar active site of the Carlsberg enzyme. Z-Ala-Ala-Cys(Bzl) is an exceptionally good inhibitor for both enzymes. As with CT the phenyl ring of Cys(Bzl) is probably inserted more deeply into the S_1 crevice. But in contrast to CT, the phenyl ring of Cys(Bzl) must now sit in a position for optimal interaction: a gain in $\Delta F°$ of -2.5 and -1.9 kcal/mol, respectively, is connected with this movement of the phenyl ring. Pattabiraman and Lawson (1972) compared the catalytic constants for the hydrolysis of $C_6H_5(CH_2)_nCH(NHCOCH_3)$-$COOCH_3$ by CT and S. BPN'. Highest values for k_{cat}/K_m(app) were found for $n = 2$ with CT but for $n = 4$ with S. BPN'. In the light of our findings, this preferential hydrolysis of long chain substrates by S. BPN' could, indeed, be caused by better substrate binding, as has been suggested by the authors. However, increased binding could not be deduced from variance in K_m(app) values which did not depend on chain length.

Excellent binding to S. Novo is also observed for the diphenylmethyl system of Cys(Dpm). But in marked contrast, S. Carlsberg can hardly accommodate this extended side chain in S_1. $\bar{K}_i$ for Z-Ala-Ala-Cys(Dpm) is the lowest value in the series tested with S. Carlsberg but the second highest with S. Novo. This now is an outstanding *qualitative* difference between the two subtilisins. We can conclude that the S_1 binding site of S. Carlsberg is more narrow than that of S. Novo which seems to associate excellently with the large diphenylmethyl group. How may this be correlated with the 3-D structure of S_1 in S. Novo and the primary structure of S. Novo and S. Carlsberg? In the crystal the aromatic substrate binding site is made up of the residues Ser-125-Leu-126-Gly-127, Ala-152-Ala-153-Gly-154, and Val-165-Gly-166-Tyr-167-Pro-168 (Wright *et al.*, 1969). Of all these residues only Val-165 is replaced by Ile in S. Carlsberg. It is hard to imagine that this replacement alone accounts for the observed differences in inhibitor binding. A more extensive conformational difference between the active sites of the two subtilisins might be deduced from fluorescence studies which indicate a distance between the side chains of His-64 to Trp-113 of only 2–5 Å for S. Carlsberg as compared to 20 Å for S. Novo (R. S. Roche and M. Shinitzky, personal communication).

Two more kinetic observations point to a different topography of the active sites of the two subtilisins. We recently reported about the different inhibition pattern of one and the same inhibitor toward different substrates and discussed the findings as evidence for two different productive substrate binding sites (Bosshard, 1973a). One site, the A site according to Wright (1972), accommodates peptide substrates, the other site (B site) the acylating agent TCI. We have extended the previous experiments including now the inhibitors Z-Ala-Ala-Cys(Dpm) and Z-Ala-Ala-Cys(Bzl) which are strictly competitive toward peptide substrates bound to the A site. Both peptides inhibit TCI hydrolysis by S. Novo in the mixed type mode characterized by increased K_m(app) which in this context means weakened substrate binding, and by lowered V_{max}. With S. Carlsberg, however, Z-Ala-Ala-Cys-(Dpm) is found to decrease K_m(app) ($\alpha > 1$ in eq 7 and 8)

together with lowering V_{max}. As regards substrate binding, this could imply a favorable interaction between TCI and the diphenylmethyl side chain of the inhibitor. Such an effect, clearly not observable with S. Novo, must point to a different topography within the active site region of S. Carlsberg.

The second observation is the inhibition pattern displayed by Bz-Arg which occupies the B site of S. Novo as does TCI (Wright, 1972). Bz-Arg inhibits noncompetitively the hydrolysis of Ac-Ala-Ala-Ala-OMe and Ac-Ala-Ala-Phe-Ala by S. Novo (Bosshard, 1973a; and unpublished experiments). In contrast, peptide ester hydrolysis by S. Carlsberg is inhibited competitively by Bz-Arg (Glazer, 1967; Bosshard, 1973a). This behavior is again best explained by a different binding mode of Bz-Arg on the two enzymes. On S. Novo Bz-Arg occupies only the B site as is evident from the X-ray work (Wright, 1972). In particular, the peptide NH of Bz-Arg and the backbone C=O of Asn-218 probably form a hydrogen bond, and the side chain of Try-217 lies parallel and in van der Waals contact to the guanidinium group of Bz-Arg. In S. Carlsberg, Tyr-217 is replaced by Leu. An additional replacement in the B site is Leu-209 in S. Novo against Tyr in S. Carlsberg (C. S. Wright, personal communication). These differences could account for a less favorable interaction of Bz-Arg with the B site of S. Carlsberg. In other words, the competitive inhibition pattern observed in the system S. Carlsberg–Bz-Arg–peptide substrate could indicate binding of Bz-Arg in the A site, with probably the benzoyl group in the S_1 crevice.

The specificity determining S_1 sites of CT and the two subtilisins are best compared from Figure 4. Small changes in the P_1 side chain are manifested much more with CT than with the subtilisins. Especially prominent is Phe *vs.* Phe($4NO_2$) and Phe($4CH_3$). Enlargement from phenyl to indole or naphthalene gives hardly a difference in binding energy for the subtilisins but increases binding by -1 kcal/mol or more for CT. Finally, Cys(Bzl), though bound well to S_1 of CT, gives much less of an increase in binding energy than is observed with both subtilisins. So far, the data correlate well with earlier observations about the subtilisins showing lower substrate specificity than CT and possessing a more extended and more open aromatic binding site (S_1 "pocket" *vs.* S_1 "crevice," Wright *et al.*, 1969). Conclusions were previously drawn from the comparison of catalytic constants for many different substrates. This leads to the important question of how similar the binding of tripeptide inhibitors is to the binding of real substrates. In experimental terms would the comparison of the catalytic constants for a series such as Z-Ala-Ala-P_1-OMe lead to the same conclusions as developed here for the series of inhibitors? It is certainly true for a number of substrates that the better the hydrophobic interaction between enzyme and substrate the higher the specificity in terms of catalysis (Knowles, 1965). But several data from the literature suggest that this simple correlation does not hold for every substrate. For example it has been reported that any substitution larger than a hydroxyl group in the para position of the phenyl ring of P_1 abolishes catalysis. Even the methyl ether derivative Ac-Tyr(CH_3)-OMe is not cleaved by CT (Kunda *et al.*, 1972). We conclude therefore that an ester of Tyr(Bzl), though presumably well bound to the enzyme, would never be hydrolyzed by CT. Other examples are iodinated tyrosines which were shaped as substrates but proved to be inhibitors (Ac-Tyr(31)-OEt has $K_i = 2200\ M^{-1}$ (Garratt and Harrison, 1970)). Ac-Cys(Bzl)-OEt may also be mentioned in this context. It is a "poorer" substrate than Ac-Phe-OMe (k_{cat}/K_m(app) = 6280 $sec^{-1}\ M^{-1}$ *vs.* 67,000 $sec^{-1}\ M^{-1}$, Damoglou *et al.*, 1970). In these and related examples the binding of the substrate, although often of high binding energy as suggested from the inhibition by virtual substrates, may lead to some distortion or misfit in the substrate–enzyme interaction at the catalytic locus which, partially or completely, abolishes catalysis. On the other hand, a lower binding energy due to strain exerted upon a substrate may in rare instances even enhance catalysis by reducing the energy barrier for the formation of a rate-limiting intermediate (Atlas and Berger, 1972). Thus, our virtual substrates are excellent probes for comparative mapping in remarkable detail of the binding sites of the three serine enzymes, but any extrapolation from these inhibition studies to the enzyme's substrate specificities have to be drawn with extreme caution. The obvious reason is that catalysis requires very accurate positioning of the substrate molecule with respect to the catalytic functionalities of the enzyme. The details and implications of this accommodation and/or adaptation of the substrates to the enzyme and *vice versa* are under well known dispute. A careful comparison of catalytic and binding parameters for a series of substrates and their corresponding virtual substrates (inhibitors), as suggested above, might shed some light on the delicate relationships between nonproductive binding, productive binding, and catalysis.

References

Abderhalden, E., and Brossa, G. A. (1909), *Chem. Ber. 42*, 3411.

Abramowitz, N., Schechter, I., and Berger, A. (1967), *Biochem. Biophys. Res. Commun. 29*, 862.

Atlas, D., and Berger, A. (1972), *Biochemistry 11*, 4719.

Aune, K. C., and Timasheff, S. N. (1971), *Biochemistry 10*, 1609.

Barel, A. O., and Glazer, A. N. (1968), *J. Biol. Chem. 243*, 1344.

Bender, M. L., Begué-Cantón, M. L., Blakeley, R. L., Brubacher, L. J., Feder, J., Bunter, C. R., Kézdy, F. J., Killheffer, J. V., Marshall, T. H., Miller, G. C., Roeske, R. W., and Stoops, K. J. (1966), *J. Amer. Chem. Soc. 88*, 5890.

Bender, M. L., Clement, G. E., Kézdy, F. J., and Heck, H. (1964), *J. Amer. Chem. Soc. 86*, 3680.

Bender, M. L., and Kemp, K. C. (1957), *J. Amer. Chem. Soc. 79*, 116.

Bergel, F., and Stock, J. A. (1954), *J. Chem. Soc.*, 2409.

Berger, A., and Schechter, I. (1970), *Phil. Trans. Roy. Soc. London, Ser. B 257*, 249.

Berger, A., Schechter, I., Benderly, H., and Kurn, N. (1971), *Peptides, Proc. Eur. Symp., 10th, 1969*, 290.

Berger, A., Smolarsky, M., Kurn, N., and Bosshard, H. R. (1973), *J. Org. Chem. 38*, 457.

Bernhard, S. A., Lee, B. F., and Tashjian, Z. H. (1966), *J. Mol. Biol. 18*, 405.

Boissonnas, R. A., Guttmann, S., Jaquenoud, P. A., and Waller, J. P. (1956), *Helv. Chim. Acta 39*, 1421.

Bosshard, H. R. (1973a), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 30*, 105.

Bosshard, H. R. (1973b), *Isr. J. Chem.* (in press).

Bosshard, H. R., and Berger, A. (1973), *Helv. Chim. Acta 56*, 1838.

Bosshard, H. R., Schechter, I., and Berger, A. (1973), *Helv. Chim. Acta 56*, 717.

Damoglou, A. P., Lindley, H., and Stapleton, I. W. (1970), *Biochem. J. 118*, 553.

Dixon, M. (1953), *Biochem. J. 55*, 161.

Faller, L. D., and LaFond, R. E. (1971), *Biochemistry 10*, 1033.

Fastrez, J., and Fersht, A. R. (1973), *Biochemistry 12*, 1067.
Foster, R. J., and Niemann, C. (1955), *J. Amer. Chem. Soc. 77*, 3365, 3370.
Gammon, K. L., Smallcombe, S. H., and Richards, J. H. (1972), *J. Amer. Chem. Soc. 94*, 4573.
Garratt, C. J., and Harrison, D. M. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 11*, 17.
Glazer, A. N. (1967), *J. Biol. Chem. 242*, 433.
Glazer, A. N. (1968), *J. Biol. Chem. 243*, 3693.
Hartley, B. S. (1970), *Phil. Trans. Roy. Soc. London, Ser. B 257*, 77.
Hayashi, Y., and Lawson, W. B. (1969), *J. Biol. Chem. 244*, 4158.
Hinkle, P. M., and Kirsch, J. F. (1971), *Biochemistry 10*, 2717.
Hirschfelder, J. O. (1965), *in* Molecular Biophysics, Pullman, B., and Weissbluth, M., Ed., New York, N. Y., Academic Press, p 325.
Hiskey, R. G., and Adams, J. B. (1965), *J. Org. Chem. 30*, 1340.
Johnson, C. H., and Knowles, J. R. (1966), *Biochem. J. 101*, 56.
Knowles, J. R. (1965), *J. Theor. Biol. 9*, 213.
Kraut, J. (1971), *Enzymes, 3rd Ed. 3*, 547.
Kundu, N., Roy, S., and Maenza, F. (1972), *Eur. J. Biochem. 28*, 311.
Laidler, K. J. (1958), The Chemical Kinetics of Enzyme Action, Oxford, Clarenden Press, p 79.
Markland, F. S., and Smith, E. L. (1971), *Enzymes, 3rd Ed. 3*, 561.
McClure, W. O., and Edelman, G. M. (1967), *Biochemistry 6*, 559.
Morihara, K., Oka, T., and Tsuzuki, H. (1969), *Biochem. Biophys. Res. Commun. 35*, 210.
Pattabiraman, T. N., and Lawson, W. B. (1972), *Biochem. J. 126*, 645.
Robertus, J. D., Alden, R. A., Birktoft, J. J., Kraut, J., Powers, J. C., and Wilcox, P. E. (1972a), *Biochemistry 11*, 2439.
Robertus, J. D., Alden, R. A., and Kraut, J. (1971), *Biochem. Biophys. Res. Commun. 42*, 334.
Robertus, J. D., Kraut, J., Alden, R. A., and Birktoft, J. J. (1972b), *Biochemistry 11*, 4293.
Schechter, I., and Berger, A. (1967), *Biochem. Biophys. Res. Commun. 27*, 157.
Schonbaum, G. R., Zerner, B., and Bender, M. L. (1961), *J. Biol. Chem. 236*, 2930.
Schwarz, H., and Bumpus, F. M. (1959), *J. Amer. Chem. Soc. 81*, 890.
Segal, D. M. (1972), *Biochemistry 11*, 349.
Segal, D. M., Powers, J. C., Cohen, G. H., Davies, D. R., and Wilcox, P. E. (1971), *Biochemistry 10*, 3728.
Smith, E. L., DeLange, R. J., Evans, W. H., Landon, M., and Markland, F. S. (1968), *J. Biol. Chem. 243*, 2184.
Steitz, T. A., Henderson, R., and Blow, D. M. (1969), *J. Mol. Biol. 46*, 337.
Teichberg, V. I., and Shinitzky, M. (1973), *J. Mol. Biol. 74*, 519.
Veber, D. F., Milkowski, J. D., Varga, S. L., Denkewalter, R. G., and Hirschmann, R. (1972), *J. Amer. Chem. Soc. 94*, 5456.
Wallace, R. A., Kurtz, A. N., and Niemann, C. (1963), *Biochemistry 2*, 824.
Wood, J. L., and duVigneaud, V. (1939), *J. Biol. Chem. 130*, 109.
Wright, C. S. (1972), *J. Mol. Biol. 67*, 151.
Wright, C. S., Alden, R. A., and Kraut, J. (1969), *Nature (London) 221*, 235.
Wünsch, E., Fries, G., and Zwick, A. (1958), *Chem. Ber. 91*, 542.

Effect of Dimethyl Sulfoxide on the Interaction of Proflavine with α-Chymotrypsin†

Anthony L. Fink

With the Technical Assistance of Mr. David E. Griffith

ABSTRACT: The effect of dimethyl sulfoxide on the binding of the competitive inhibitor proflavine to α-chymotrypsin has been investigated as a function of dimethyl sulfoxide concentration. The change in K_p (the dissociation constant for the α-chymotrypsin–proflavine complex) with increasing dimethyl sulfoxide concentration exactly parallels the previously observed increase in K_m (A. L. Fink (1973), *Biochemistry 12*, 1736) indicating that the effect on K_m reflects a change in K_s, *i.e.* substrate binding. The temperature dependence of K_p was found to be negligible. The effect of dimethyl sulfoxide on K_m and K_p could be quantitatively accounted for by a combination of competitive inhibition and dielectric effects by dimethyl sulfoxide on substrate and inhibitor binding.

In connection with our investigations of enzyme-catalyzed reactions at subzero temperatures and in aqueous–organic solvent systems, we have observed that the main difference in the reaction under such conditions compared with normal conditions is an increase in K_m. For example, we have observed a linear relationship between log K_m and dimethyl sulfoxide concentration in the α-chymotrypsin–aqueous dimethyl sulfoxide system (Fink, 1973). This increase in K_m was tenta-

† From the Division of Natural Science, University of California, Santa Cruz, California 95604. *Received July 26, 1973.* This research was supported by a grant from the National Science Foundation.