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THE ACTIVE CENTERS OF *STREPTOMYCES GRISEUS* PROTEASE 3 AND α-CHYMOTRYPSIN

ENZYME-SUBSTRATE INTERACTIONS BEYOND SUBSITE S_1'

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Summary

A series of *N*-acetylated tetra- to heptapeptide amides has been synthesized for the study of enzyme-substrate interactions beyond the S_1' subsite in *Streptomyces griseus* Protease 3 (SGP3) and α-chymotrypsin (EC 3.4.21.1). Evidence was obtained that S_2'-P_2' enzyme-substrate interactions can play a significant role for the rate of substrate hydrolysis in both enzymes. No important interaction could be demonstrated beyond the nitrogen atom of residue P_3'. This provides supplementary evidence that the active site of SGP3 extends over 6—7 subsites and that of α-chymotrypsin over 5—6 subsites.

SGP3 is a considerably more efficient protease than α-chymotrypsin, k_{cat}/K_m being approximately $5 \cdot 10^6$ $s^{-1} \cdot M^{-1}$ for the best substrates, thus being about 100 times higher than for α-chymotrypsin. However, an analysis of the kinetic data indicates that, for both enzymes, the acylation rates for the best peptide substrates approach their deacylation rates.

Introduction

The size of the active site of several proteases has been shown to extend over many subsites. For example, the active sites of papain and elastase have been shown to extend over 6—7 subsites (21—25 Å) [1,2] and that of carboxypeptidase A over about 5 subsites (approx. 18 Å) [3]. Extended active sites are important both for increasing enzyme-substrate affinities and turnover rates. So far, most studies on the importance of extended active sites have explored enzyme-substrate interactions remote from, and N-terminal to, the scissile bond. An example of an enzyme which for its activity is highly dependent on such remote interactions is pancreatic elastase [4,5]. In contrast, α-chymotrypsin (EC 3.4.21.1), although possessing an extended active site, shows compara-

tively low dependence on remote interactions, occurring N-terminal to the scissile bond [6—9]. Recently, however, it was found that a microbial enzyme, *Streptomyces griseus* Protease 3 * (SGP3), which has a specificity similar to that of α-chymotrypsin [17] shows a most prominent dependence on remote interactions N-terminal to the scissile bond [9].

S_1' - P_1' ** interactions are also important in increasing the rate of substrate hydrolysis by, for example α-chymotrypsin [17—19], elastase [4,17] and SGP3 [17], but the importance of enzyme-substrate interactions remote from and C-terminal to the scissile bond is only poorly understood. However, since these enzymes are all endopeptidases, it appears probable that enzyme-substrate interactions beyond subsite S_1' may also be of importance for hydrolysis. Indeed, for α-chymotrypsin there are indications that such interactions may contribute to the rate of hydrolysis [6,7].

The aim of the present study is to prepare peptide substrates suitable for studying the importance of enzyme-substrate interactions beyond subsite S_1' in α-chymotrypsin and SGP3 and to estimate the length of the active sites of these enzymes.

Materials and Methods

α-Chymotrypsin was a triply crystallized preparation from Worthington. Homogeneous lyophilized SGP3 was prepared from Pronase-P as described by Bauer and Löfqvist [11]. The concentrations of active enzymes were determined as described by Bauer et al. [9].

Hydrolysis of the peptides was carried out in a pH-stat under a N_2 atmosphere at 37°C in 1 ml of 10 mM $CaCl_2$, 3 mM NaN_3 at pH 8.00 (α-chymotrypsin) or pH 9.00 (SGP3). The concentrations of active enzymes during the assays were 0.56—0.023 μM (α-chymotrypsin) and 0.25—0.0022 μM (SGP3).

$pKa_{NH_3} = 8.90$, $pKa_{H\text{-}Ala\text{-}NH_2} = 7.78$, $pKa_{H\text{-}Ala_2\text{-}NH_2} = 7.72$, $pKa_{H\text{-}Ala_3\text{-}NH_2} = 7.67$ were all determined titrimetrically under the conditions used in the substrate assays. Product ionization was corrected according to Kurtz and Niemann [20].

The amine product of hydrolysis is a buffer in the pH region of interest and can potentially influence the step size of the titrimeter [8,21]. Like in our earlier investigations [9,17], no corrections have been made for this effect, since in all cases initial rates have been measured, and the concentration of the buffering product is, therefore, low. We have never observed any significant changes in the step size during this initial phase of a reaction.

The parameters, k_{cat} and K_m of the Michaelis-Menten equation and their

* SGP3 has been shown [10,11] to be identical to "PNPA-hydrolase I", described by Wählby [12], "alkaline protease a" (Narahashi [13]), "*Streptomyces griseus* Enzyme II" (Gertler and Trop [14]), "*Streptomyces griseus* protease A" (Johnson and Smillie [15]), and "lysine-free chymoelastase" (Siegel and Awad [16]).

** The nomenclature introduced by Schechter and Berger [1] is used to facilitate discussion of the interactions between a protease and bound peptides. Amino acid residues and partial amino acid residues (e.g., acetyl groups) of substrates are numbered P_1, P_2, P_3, etc., in the N-terminal direction, and P_1', P_2', etc., in the C-terminal direction from the scissile bond. The complementary subsites of the enzyme's active center are numbered S_1, S_2 and S_1', S_2', etc., in an analogous fashion. The binding mode of a peptide which occupies, for example, the S_4, S_3, S_2, and S_1 subsites of the enzyme will be denoted by the abbreviation S_{4321}.

standard deviations were determined by iterative regression analysis [22]. Strict Michaelis-Menten kinetics were observed. Preliminary parameter estimates, necessary for the iterative regression analysis, were obtained graphically from Lineweaver-Burke plots.

The bond split by the enzymes was established by thin-layer chromatography of reaction products. In all the present cases, results were consistent with cleavage of the Phe-NH_2 or the Phe-Ala bonds, only.

Thin-layer chromatography of peptides was carried out with silica gel plates (Merck), which were developed in *n*-butanol/acetic acid/water (4 : 1 : 1). Spots were visualized by spraying with a 0.1% ninhydrin spray reagent (Merck) and heating at 110°C for 5 min. *t*-Butyloxycarbonyl peptides could generally be visualized by prolonged heating (60 min). After ninhydrin visualization, the plates were exposed to chlorine vapour for some minutes and sprayed with an aqueous solution of KI and tolidine (Reagent 32 in ref. 23).

Amino acid derivatives were purchased from Cyclo Chemicals and Sigma Chemical Company. All amino acids are of the L-configuration. The preparations of Ac-Pro-Ala-Pro-OH [5], Ac-Pro-Ala-Pro-Phe-NH_2 (I) [9] and Ac-Pro-Ala-Pro-Phe-Ala-NH_2 (II) [17] have all been described earlier.

General methods of preparation

Coupling procedure. *n* mmol of N-protected amino acid or peptide were dissolved in *N,N*-dimethylformamide and cooled to −20°C in a solid CO_2-CCl_4 bath, *N*-methylmorpholine (*n* mmol) was added, followed by isobutyl chloroformate (*n* mmol) after 5 min. After 3 min a precooled solution of *n* mmol peptide amide hydrochloride in *N,N*-dimethylformamide was added, immediately followed by *n* mmol *N*-methylmorpholine, and the solution stirred overnight, The solvent was evaporated in vacuo and the residue, if reasonably water soluble, was dissolved in water and treated with an excess of Rexyn I-300 resin (Fisher), filtered and the water evaporated. In cases where the product was poorly water soluble, it was dissolved in chloroform and extracted twice with 0.2 M HCl, twice with 5% aqueous $NaHCO_3$ and once with water. The organic phase was dried and evaporated. After one of these two alternative purification methods, the residue was crystallized as stated for each compound.

Deprotection procedure. A *N-tert*-butyloxycarbonyl (*t*-Boc) protected peptide amide was added to ethyl acetate in a round-bottom flask and cooled in an ice bath. Hydrogen chloride was bubbled through the solution for 15 min. During the first few minutes the reaction mixture warmed up, but heat soon ceased to be produced, the ice bath was then removed and the reaction mixture allowed to warm to room temperature. After 1 h the solvent was removed in vacuo and the residue left in vacuo over NaOH pellets overnight. The residue was crystallized as described below.

Synthetic steps in the preparation of Ac-Pro-Ala-Pro-Phe-Ala-Ala-NH_2 (III)

A. Synthesis of t-Boc-Phe-Ala-Ala-NH_2. The compound was synthesized from *t*-Boc-Phe-OH (10 μmol/ml) and HCl · H-Ala-Ala-NH_2 (10 μmol/ml) by the coupling procedure described above. After purification by extraction, the compound was crystallized from ethyl acetate giving a yield of 100 mg (44%) of

crystals of m.p. 199—201°C, and a single spot by thin-layer chromatography $R_{F_{II}}$ 0.75.

B. Synthesis of HCl · H-Phe-Ala-Ala-NH₂. After deprotection of *t*-Boc-Phe-Ala-Ala-NH_2 (5 μmol/ml), as described above, the compound was crystallized from acetone to give 57 mg (71%) of crystals of m.p. 226—228°C, and a single spot by thin-layer chromatography $R_{F_{II}}$ 0.25.

C. Synthesis of Ac-Pro-Ala-Pro-Phe-Ala-Ala-NH₂ (III). The compound was prepared by a coupling reaction using Ac-Pro-Ala-Pro-OH (6 μmol/ml) and HCl · H-Phe-Ala-Ala-NH_2 (6 μmol/ml). After purification with Rexyn I-300 the peptide was crystallized from hot ethyl acetate, to give 60 mg (58%) of white crystals of m.p. 195—199°C and showing a single tolidine positive spot by thin-layer chromatography $R_{F_{II}}$ 0.2.

Amino acid analysis: theoretical: Ala, 3.00; Phe, 1.00; Pro, 2.00. Found: Ala, 2.80; Phe, 0.95; Pro, 2.00.

Synthetic steps in the preparation of Ac-Pro-Ala-Pro-Phe-Ala-Ala-Ala-NH₂ (IV)

A. Synthesis of t-Boc-Ala-Ala-Ala-NH₂. The compound was synthesized from *t*-Boc-Ala-OH (12 μmol/ml) and HCl · H-Ala-Ala-NH_2 (12 μmol/ml), and purified with Rexyn I-300. Crystallization from acetone/ethyl acetate (1 : 20) gave 80 mg (70%) of white crystals. m.p. 209—211°C, single spot by thin-layer chromatography $R_{F_{II}}$ 0.5.

B. Synthesis of HCl · H-Ala-Ala-Ala-NH₂. After deprotection of *t*-Boc-Ala-Ala-Ala-NH_2 (10 μmol/ml) by the method above, the compound was crystallized from 5% aqueous methanol/acetone (1 : 10). Yield of white crystals 55 mg (86%) of m.p. 243—245°C and a single spot by thin-layer chromatography $R_{F_{II}}$ 0.05.

C. Synthesis of t-Boc-Phe-Ala-Ala-Ala-NH₂. The compound was prepared by a coupling reaction using *t*-Boc-Phe-OH (10 μmol/ml) and HCl · H-Ala-Ala-Ala-NH_2 (10 μmol/ml), and purified by extraction. Crystallization from ethyl acetate yielded 57 mg (58%) of m.p. 211—212°C, and a single spot by thin-layer chromatography $R_{F_{II}}$ 0.7.

D. Synthesis of HCl · H-Phe-Ala-Ala-Ala-NH₂. *t*-Boc-Phe-Ala-Ala-Ala-NH_2 (10 μmol/ml) was deprotected as described above. The solid residue was crystallized from acetone/ethyl acetate, giving 40 mg (81%) of crystals of m.p. 227—229°C and a single spot by $R_{F_{II}}$ 0.2.

E. Synthesis of Ac-Pro-Ala-Pro-Phe-Ala-Ala-Ala-NH₂ (IV). The compound was synthesized from Ac-Pro-Ala-Pro-OH (5 μmol/ml) and HCl · H-Phe-Ala-Ala-Ala-NH_2 (5 μmol/ml) as described above, and purified with Rexyn I-300. Crystallization from boiling ethyl acetate gave 30 mg (55%) of white crystals of m.p. 229—232°C and a single tolidine-positive spot by thin-layer chromatography $R_{F_{II}}$ 0.15.

Amino acid analysis: theoretical: Ala, 4.00; Phe, 1.00; Pro, 2.00. Found: Ala, 3.81; Phe, 0.89; Pro, 2.00.

Results and Discussion

The hydrolysis of the peptides I—IV is restricted to a single peptide or amide bond, as shown both by product analysis using thin-layer chromatography and

by the uptake of only one equivalent (± 5%) of base per mol of substrate during the hydrolysis. This indicates that there will be one productive binding mode, only.

For peptides of the sequence Ac-Pro-Ala-Pro-Phe-X it is likely that the only strong binding mode is S_{543211}', because the S_3 subsites of SGP3 and α-chymotrypsin appear unable to bind prolyl residues (for a discussion, see [9,17]). With the hexapeptide and especially with the heptapeptide, which both have longer sequences without prolyl residues, nonproductive binding may be significant. If nonproductive binding was more pronounced with peptide IV than with peptide III, K_m and k_{cat} would be lower for IV. As can be seen from Tables I and II, this is not the case. In addition, the binding energies observed for various different peptides to SGP3 and α-chymotrypsin [9,17], in relation to the present K_m values for III and IV, indicate that nonproductive binding of the latter peptides would be kinetically insignificant. It is reasonable to believe, therefore, that the only strong binding modes for peptides III and IV are $S_{543211'2'}$ and $S_{543211'2'3'}$, respectively.

It has earlier been shown that there is about a 30-fold increase in k_{cat}/K_m for SGP3 (Table I) and about a 15-fold increase for α-chymotrypsin (Table II) on going from peptide I to peptide II [17]. The synthesis of peptides III and IV has now made it possible to study enzyme-substrate interactions beyond the S_1' subsites, by virtue of the unique binding mode of these substrates. For both enzymes there is an increase in k_{cat}/K_m on extending the pentapeptide (II) to a hexapeptide (III) with a P_2' alanyl residue (Tables I and II). While this increase is about 15-fold for SGP3, it is only 4-fold for α-chymotrypsin. No obvious increase in k_{cat}/K_m results from further elongating the hexapeptide (III) to the

TABLE I

KINETIC PARAMETERS FOR SGP3-CATALYZED HYDROLYSIS OF POLYPEPTIDES AT pH 9.00

P_5 P_4 P_3 P_2 P_1 ↓ P_1' P_2' P_3' P_4'		$k_{cat}/K_m(s^{-1}M^{-1})$	$k_{cat}(s^{-1})$	K_m(mM)	[S]* (mM)
Ac-Pro-Ala-Pro-Phe-NH_2 **	(I)	10 700	5.8 ± 0.1	0.54 ± 0.03	0.47—7.1
Ac-Pro-Ala-Pro-Phe-Ala-NH_2 **	(II)	310 000	40.0 ± 0.7	0.13 ± 0.01	0.23—3.7
Ac-Pro-Ala-Pro-Phe-Ala-Ala-NH_2	(III)	4 700 000	250 ± 4	0.053 ± 0.005	0.12—0.93
Ac-Pro-Ala-Pro-Phe-Ala-Ala-Ala-NH_2	(IV)	5 600 000	280 ± 4	0.050 ± 0.006	0.12—0.93

* Range of substrate concentrations.
** Data from Bauer et al. [17].

TABLE II

KINETIC PARAMETERS OF α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF POLYPEPTIDES AT pH 8.00

P_5 P_4 P_3 P_2 P_1 ↓ P_1' P_2' P_3' P_4'		$k_{cat}/K_m(s^{-1}M^{-1})$	$k_{cat}(s^{-1})$	K_m(mM)	[S]* (mM)
Ac-Pro-Ala-Pro-Phe-NH_2 **	(I)	820	2.8 ± 0.2	3.4 ± 0.4	0.93—7.5
Ac-Pro-Ala-Pro-Phe-Ala-NH_2 **	(II)	11 700	18.7 ± 0.9	1.6 ± 0.2	0.77—3.6
Ac-Pro-Ala-Pro-Phe-Ala-Ala-NH_2	(III)	44 000	36.6 ± 1.7	0.83 ± 0.09	0.24—1.9
Ac-Pro-Ala-Pro-Phe-Ala-Ala-Ala-NH_2	(IV)	45 000	37.0 ± 2.9	0.82 ± 0.15	0.24—1.9

* Range of substrate concentrations.
** Data from Bauer et al. [17].

heptapeptide (IV). There is a remarkable difference in efficiency between SGP3 and α-chymotrypsin, in that SGP3 hydrolysis peptides III and IV about 100-fold more efficiently than α-chymotrypsin. This is reflected in K_m being about 15-fold lower and k_{cat} about 7-fold higher for SGP3. For both enzymes, however, the k_{cat} values for III and IV appear so high that the usual approximation $k_{cat} \approx k_2$ for peptide and amide hydrolysis [24] may not hold. The kinetics of the present reactions will, therefore, be discussed briefly.

Substrate hydrolysis by α-chymotrypsin [24] and SGP3 [25] can be described by the kinetic scheme

$$E + S \underset{K_s}{\rightleftharpoons} ES \underset{P_1}{\overset{k_2}{\rightarrow}} EA \overset{k_3}{\rightarrow} E + P_2$$

where E is free enzyme, S, substrate, ES enzyme-substrate complex, EA acyl-enzyme and P_1 and P_2 products.

Zerner and Bender [24] have shown that where the K_s equilibrium is established much more rapidly than acylation, the Michaelis constant, K_m, is related to the dissociation constant of the productive enzyme substrate complex, K_s, by

$$K_m = \frac{k_3 K_s}{k_2 + k_3} \tag{1}$$

and that the turnover rate, k_{cat}, is related to the rate constants, k_2 and k_3 by

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3} \tag{2}$$

For peptidase and amidase activity it has been shown that, generally, $k_2 \ll k_3$, reducing Eqns. 1 and 2 to $K_m \approx K_s$ (Eqn. 3) and $k_{cat} \approx k_2$ (Eqn. 4) [24]. To test whether $k_2 \ll k_3$ for the peptides I—IV, it is necessary to know the magnitude of k_3. This can be determined from substrates which form the same acyl-enzyme with SGP3 and α-chymotrypsin * as the peptides and for which deacylation is rate-determining. Ac-Pro-Ala-Pro-Phe methyl ester appears to be a suitable substrate for such a determination, since there is evidence that deacylation is the rate-determining step for the hydrolysis of this substrate by both enzymes [28]. With this substrate, $k_{cat} = 95\ s^{-1}$ for α-chymotrypsin and $k_{cat} = 750\ s^{-1}$ for SGP3 [28]. Since the deacylation rate constant is the same for the peptides and Ac-Pro-Ala-Pro-Phe methyl ester, K_s and k_2 can now be calculated from Eqns. 1 and 2, and the values are tabulated in Table III.

The small decrease in K_s on going from peptide II to peptide III (Table III) indicates that subsite S_2', in both enzymes, is a rather weak binding site for an alanyl residue, or that some of the potential free energy of binding is used to reduce the free energy of activation. The acylation rates increase 2- to 3-fold in α-chymotrypsin but almost 10-fold in SGP3. Further elongation of the substrate chain to peptide IV has no significant effect on K_s or k_{cat} in either α-chymotrypsin or SGP3. The similarity of the kinetic constants for peptides

* It has been shown that an acyl-enzyme is formed during α-chymotrypsin catalyzed hydrolysis of esters, amides and peptides [24,26,27].

III and IV, therefore, indicates that, in both enzymes, there is probably no important enzyme-substrate interaction beyond the nitrogen atom of residue P_3'. It may also be pointed out that, for both enzymes, the acylation rates (k_2) for the best substrates tested here (peptides III and IV), rather unexpectedly approach their deacylation rates (k_3) (cf. Table III).

Recently, it was shown that the acylation rate increases about 800-fold in SGP3, but only about 10-fold in α-chymotrypsin on going from Ac-Phe-NH_2 to Ac-Pro-Ala-Pro-Phe-NH_2 [9]. On extending the substrate chain in the C-terminal direction it has now been shown that the acylation rate increases almost 80-fold in SGP3 and about 20-fold in α-chymotrypsin on going from peptide I to IV. SGP3 is thus more sensitive to chain length than α-chymotrypsin on the "C-terminal side", also, although this sensitivity is not as dramatic as that on the "N-terminal side", at least not for long peptides. It is of course quite possible that the S'-P'interactions would appear relatively more important if all the S-P interactions were not formed.

Determination of the exact length of the active site of a protease with the help of specific substrates presents a number of problems. For example, the results presented here strongly indicate that there is no important enzyme-substrate interaction beyond the nitrogen atom of residue P_3'. However, it is not easy to tell if the ultimate interaction is between the enzyme and the P_2' carbonyl group or the P_3' amide group, or even if the ultimate interaction is between the P_2' side chain and the enzyme. Trying to solve the former problem with the use of an unamidated P_2' residue does not seem to be a good solution, since a free C-terminal carboxyl group will be charged at the pH values used, and this charge can introduce new interactions [19], making interpretation of the results impossible.

The results presented here, together with those of a previous investigation [9], suggest that the active site of SGP3 does not extend over more than 7 subsites and that of α-chymotrypsin not over more than 6 subsites. The results do not, however, exclude the possibility that the active sites of the enzymes are about one subsite shorter than those values. If the polypeptide substrate is assumed to bind in an extended form with a length of 3.5 Å per residue [1], this

TABLE III

CALCULATED K_s, k_2 and k_3 VALUES FOR SGP3 AND α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF POLYPEPTIDES (CF. TABLES I AND II).

P_5-P_4 P_3 P_2 P_1 ↓ P_1' P_2' P_3' P_4'	SGP3			α-chymotrypsin		
	K_s(mM)	$k_2(s^{-1})$	$k_3^*(s^{-1})$	K_s(mM)	$k_2(s^{-1})$	$k_3^*(s^{-1})$
Ac-Pro-Ala-Pro-Phe-NH_2 (I)	0.544	5.85	750	3.50	2.88	95
Ac-Pro-Ala-Pro-Phe-Ala-NH_2 (II)	0.137	42.3	750	1.99	23.3	95
Ac-Pro-Ala-Pro-Phe-Ala-Ala-NH_2 (III)	0.0795	375	750	1.35	59.5	95
Ac-Pro-Ala-Pro-Phe-Ala-Ala-Ala-NH_2 (IV)	0.0798	447	750	1.34	60.6	95

* The k_3 values are, in both cases, based on k_{cat} values for Ac-Pro-Ala-Pro-Phe methyl ester [28].

means that the active site of SGP3 extends over 21—25 Å and that of α-chymotrypsin over 18—21 Å.

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References

1 Schechter, I. and Berger, A. (1967) Biochem. Biophys. Res. Commun. 27, 157—162
2 Atlas, D., Levit, S., Schechter, I. and Berger, A. (1970) FEBS Lett. 11, 281—283
3 Abramowitz, N., Schechter, I. and Berger, A. (1967) Biochem. Biophys. Res. Commun. 29, 862—867
4 Thompson, R.C. and Blout, E.R. (1970) Proc. Natl. Acad. Sci. U.S. 67, 1734—1740
5 Thompson, R.C. and Blout, E.R. (1973) Biochemistry 12, 57—65
6 Yoshida, N., Yamamoto, T. and Izumiya, N. (1968) Arch. Biochem. Biophys. 123, 165—171
7 Morihara, K., Oka, T. and Tsuzuki, H. (1969) Biochem. Biophys. Res. Commun. 35, 210—214
8 Baumann, W., Bizzozero, S. and Dutler, H. (1973) Eur. J. Biochem. 39, 381—391
9 Bauer, C.-A., Thompson, R.C. and Blout, E.R. (1976) Biochemistry, 15, 1291—1296
10 Löfqvist, B. and Sjöberg, L.B. (1971) Acta Chem. Scand. 25, 1663—1678
11 Bauer, C.-A. and Löfqvist, B. (1973) Acta Chem. Scand. 27, 3147—3166
12 Wählby, S. (1969) Biochim. Biophys. Acta 185, 178—185
13 Narahashi, Y. (1970) in Methods Enzymol. 19, 651—664
14 Gertler, A. and Trop, M. (1971) Eur. J. Biochem. 19, 90—96
15 Johnson, P. and Smillie, L.B. (1971) Can. J. Biochem. 49, 548—562
16 Siegel, S. and Awad, W.M. (1973) J. Biol. Chem. 248, 3233—3240
17 Bauer, C.-A., Thompson, R.C. and Blout, E.R. (1976) Biochemistry, 15, 1296—1300
18 Baumann, W., Bizzozero, S. and Dutler, H. (1970) FEBS Lett. 8, 257—260
19 Fersht, A.R., Blow, D.M. and Fastrez, J. (1973) Biochemistry 12, 2035—2041
20 Kurtz, A.N. and Niemann, C. (1962) Biochemistry 1, 238—243
21 Jacobsen, C.F., Leonis, J., Linderstrøm-Lang, K. and Ottesen, M. (1957) Methods Biochem. Anal. 4, 171
22 Pettersson, G. and Petterson, I. (1970) Acta Chem. Scand. 24, 1275—1286
23 Waldi, D. (1965) in Thin Layer Chromatography, (Stahl, F., ed.), New York, N.Y. Springer Verlag, p. 488
24 Zerner, B. and Bender, M.L. (1964) J. Am. Chem. Soc. 86, 3669—3674.
25 Bauer, C.-A., Löfqvist, B. and Pettersson, G. (1974) Eur. J. Biochem. 41, 45—49
26 Berezin, I.V., Kazanskaya, N.F., Klyosov, A.A. and Svedas, V.K. (1973) Eur. J. Biochem. 38, 529—536
27 Fastrez, J. and Fersht, A.R. (1973) Biochemistry 12, 2025—2034
28 Bauer, C.-A., unpublished results.