Biochimica et Biophysica Acta, 614 (1980) 113—120
© Elsevier/North-Holland Biomedical Press

BBA 69021

INHIBITION OF HUMAN PANCREATIC ELASTASE 2 BY PEPTIDE CHLOROMETHYL KETONES

COREY LARGMAN [a,b], ERIC G. DELMAR [b], JAMES W. BRODRICK [a,b], MARIA FASSETT [b] and MICHAEL C. GEOKAS [a,b]

[a] *The Enzymology Reserach Laboratory, Martinez Veterans Administration Hospital, Martinez, CA 94553 and* [b] *the Department of Internal Medicine, University of California at Davis, Davis, CA 95616 (U.S.A.)*

(Received January 2nd, 1980)

Key words: Pancreatic elastase; Peptide chloromethyl ketone; Elastase inhibition; (Human)

Summary

The inactivation of human pancreatic elastase 2 (EC 3.4.21.11) by a series of peptide chloromethyl ketones has been investigated. Among a series of compounds with the structure X-Ala-Ala-Pro-Y-CH_2Cl (where X = acetyl-, succinyl-, methylsuccinyl-, or H-), the kinetic parameters for inhibition of elastase 2 depend markedly on the amino acid (Y) in the P1 position. Succinyl-Ala-Ala-Pro-Leu-CH_2Cl was found to be an extremely effective inhibitor of human elastase 2, with a first-order rate constant for covalent bond formation (k_3) of 0.033 s^{-1} and a dissociation constant, K_i, for the enzyme inhibitor complex of $7.4 \cdot 10^{-7}$ M. The second-order rate constant k_3/K_i for inhibition of elastase 2 by the analogous compound containing a free amino group in place of the succinyl moiety is 150 times lower than that found for the succinyl or acetyl derivative, suggesting that the presence of a positive charge at this position reduces the proper binding of the inhibitor to the enzyme.

Introduction

The use of specific amino acid chloromethyl ketone compounds to inhibit trypsin and chymotrypsin represented one of the original applications of the affinity labeling technique for enzyme studies [1]. Following this early work, specific peptide chloromethyl ketones have been described for inhibition of closely related trypsin-like enzymes [2] as well as for inhibition of serine proteases with specificity for neutral amino acids, such as porcine pancreatic

Abbreviations: MeOSuc, methoxysuccinyl; Cbz, carboxybenzyloxy.

elastase 1 (EC 3.4.21.11) [3,4], human leukocyte elastase [5], and bovine chymotrypsin [6]. The possible therapeutic use of peptide chloromethyl ketones was suggested by their ability to reduce lung elastin destruction in hamsters when given prior to the administration of porcine elastase 1 [7]. Human pancreatic elastase 2 has a different specificity for hydrolysis of peptide *p*-nitroanilides [8,9] than porcine pancreatic elastase 1 [10] or human leukocyte elastase [11]. The present study was designed to gain further insight into the peptide bond specificity of human pancreatic elastase 2, and to obtain a specific and efficient inhibitor for this enzyme.

Materials and Methods

Peptide chloromethyl ketones. Suc-Ala-Ala-Pro-Val-CH_2Cl and MeOSuc-Ala-Ala-Pro-Val-CH_2Cl were generous gifts from Dr. J.C. Powers, School of Chemistry, Georgia Institute of Technology, Atlanta, GA. Ac-Ala-Ala-Pro-Val-CH_2Cl, Ac-Ala-Ala-Pro-Ile-CH_2Cl, Ac-Ala-Ala-Pro-Ala-CH_2Cl, and Ac-Ala-Ala-Ala-Ala-CH_2Cl were all kindly provided by Dr. Powers under an N.I.H. contract for synthesis and distribution of elastase inhibitors.

NH_2-Ala-Ala-Pro-Leu-CH_2Cl. The diazoketone Cbz-Leu-CH_2N_2 was prepared from Cbz-leucine (5.2 g) and diazomethane by the mixed anhydride method using 1 equiv. each of isobutylchloroformate and triethylamine. The resulting diazoketone was not isolated. Anhydrous HCl was bubbled through the solution of Cbz-Leu-CH_2N_2 in diethyl ether/ethyl acetate for 1 h at 4°C and the solution turned from yellow to colorless. Following removal of solvent, the Cbz group was removed from the residual oil by treatment with 32% HBr in acetic acid. The hydrobromide salt of NH_2-Leu-CH_2Cl was isolated by washing the initial oil with petroleum ether and trituration of the residue with diethyl ether. The overall yield was 2.0 g (46%).

Cbz-Ala-Ala-Pro-Leu-CH_2Cl. Cbz-Ala-Ala-Pro was prepared as described previously [9]. This compound was coupled to NH_2-Leu-CH_2Cl by the mixed anhydride procedure using 1 equiv. of isobutylchloroformate and 2 equiv of triethylamine in methylene chloride. The reaction mixture was washed with dilute acid and then with saturated sodium bicarbonate. Following removal of solvent in vacuo, the initial oil was triturated with diethyl ether to give a white solid, m.p. = 139—141°C. The yield was 3.5 g (70%).

NH_2-Ala-Ala-Pro-Leu-CH_2Cl. Cbz-Ala-Ala-Pro-Leu-CH_2Cl (1.5 g) was dissolved in 32% HBr in acetic acid in order to remove the Cbz protecting group. The hydrobromide salt of the free amine product was precipitated with ether, and stirring at 4°C yielded a white crystalline solid. The yield was 1.94 g (144%), probably due to excess HBr associated with the product. the product gave a single spot, R_F = 0.30 by thin-layer chromatography in acetone.

Suc-Ala-Ala-Pro-Leu-CH_2Cl. This was prepared by reacting the free amine (1.9 g) with succinic anhydride (0.47 g, 10% excess) in methylene chloride with 2 equiv. of *N*-ethylmorpholine. The reaction mixture was stirred for 1 h at room temperature, extracted with acidic water and the methylene chloride fraction was concentrated in vacuo to yield an oil. The product was precipitated by addition of ether to yield 0.54 g (28%) of a white solid. The

product gave a single spot (R_F = 0.75) on thin-layer chromatography (TLC) in 50 : 50 tetrahydrofuran/methanol.

Ac-Ala-Ala-Pro-Leu-CH_2Cl. This was prepared by reacting NH_2-Ala-Ala-Pro-Leu-CH_2Cl (0.8 g) with a 10% excess of acetic anhydride in methylene chloride. The reaction mixture was washed with dilute acid and saturated sodium bicarbonate. Removal of the solvent in vacuo yielded 0.21 g (25%) of a white solid, m.p. = 137—139°C. The product gave a single spot (R_F = 0.76) by TLC in acetone.

Human pancreatic elastase 2 was purified as previously described [8].

Methods

Kinetic analysis. Elastase 2 was assayed by adding a 50 µl aliquot of an incubation mixture to 0.7 ml of Suc-Ala-Ala-Pro-Leu-*p*-nitroanilide [9] at a concentration of 1 mg/ml in 0.2 M Tris-HCl (pH 8.0). The change in absorbance at 410 nm was recorded continuously using a Gilford Model 252 spectrophotometer with a cell maintained at 25°C. Inhibition reactions were performed at 25°C in 0.1 M sodium phosphate (pH 6.85). The inhibitors were prepared as stock solutions in methanol at concentrations such that a final concentration of 1% methanol was achieved in the incubation mixture. The k_{obs} was determined for each inhibition reaction from the semilog plot of enzyme activity vs. time, using the equation $k_{obs} = \log 0.5/t_{1/2}$. The data for each determination of k_{obs} were fitted to a line by a least-squares analysis. The criteria for accepting a determination of k_{obs} was a linear regression coefficient of at least 0.97.

Determination of $k_{obs}/[I]$ values. In these experiments, human elastase 2 was employed at a concentration of $1.8 \cdot 10^{-7}$ M. Each inhibitor was added to a reaction mixture to yield a concentration in the range of 10^{-4} to $4 \cdot 10^{-6}$ M. At each of eight timed intervals, a 50 µl aliquot was removed for assay of elastase 2 activity. Duplicate determinations of k_{obs} were made at each of three inhibitor concentrations over a 10-fold range to establish that $k_{obs}/[I]$ was constant.

Determination of k_3 and K_i values. The concentration of human elastase 2 employed in these experiments was $1.8 \cdot 10^{-8}$ M. In order to increase sensitivity, 50 µl of the incubation mixture were assayed using 3.5 mg/ml Suc-Ala-Ala-Pro-Leu-*p*-nitroanilide. For each inhibitor concentration, k_{obs} was calculated from a five-point pseudo-first-order plot of log E vs. time. Duplicate determinations of k_{obs} for inhibition of elastase 2 by each inhibitor were made at a minimum of six inhibitor concentrations over a range of 10^{-7}—10^{-6} M for the acetyl and succinyl derivatives, and a range of $6.6 \cdot 10^{-6}$ to $5 \cdot 10^{-5}$ M for the free amino derivative.

Results and Discussion

The kinetic analysis of the inactivation of an enzyme by an irreversible inhibitor has been described [12]. The reaction of a chloromethyl ketone with a protease may be represented by eqn. 1, where E · I represents a noncovalently bound complex of the enzyme with the inhibitor and E-I is the final product with the inhibitor irreversibly bound to the enzyme by a covalent bond.

$$E + I \rightleftharpoons E \cdot I \overset{k_3}{\rightarrow} E - I \qquad (1)$$

When k_3 is sufficiently slow, there is an equilibrium between [E · I] and [E] + [I]. The dissociation constant K_i for this process is expressed by eqn. 2.

$$K_i = \frac{[E][I]}{[E \cdot I]} \quad (2)$$

Under conditions when [I] >> E_0, it can be shown [12,13] that the pseudo-first-order inhibition rate constant k_{obs} is related to K_i by eqn. 3.

$$1/k_{obs} = K_i/k_3[I] + 1/k_3 \quad (3)$$

When $[I]_0 << K_i$, $k_{obs}/[I]$ is constant with changing concentrations of I, and is approx. equal to k_3/K_i [13]. Thus the parameter $k_{obs}/[I]$ has been employed for comparison of the reactivity of various inhibitors with serine endopeptidases [13]. The $k_{obs}/[I]$ values for inhibition of human elastase 2 by a series of compounds of the form X-Ala-Ala-Pro-amino acid-CH_2Cl are shown in upper portion of Table I. For each of these inhibitors, $k_{obs}/[I]$ was shown to be constant over a 10-fold range of inhibitor concentration.

When K_i and [I] are similar, both k_3 and K_i can be obtained from a double reciprocal plot of $1/k_{obs}$ vs. 1/[I] as indicated by eqn. 3. However, it should be noted that when K_i is much greater than the inhibitor concentrations employed, the plot of $1/k_{obs}$ vs. 1/[I] will pass through the origin and K_i cannot be evaluated. Fig. 1 shows the plots of $1/k_{obs}$ vs. 1/[I] for the inhibition of human elastase 2 by serveral chloromethyl ketone compounds of the structure X-Ala-Ala-Pro-Leu-CH_2Cl. In each case, the line drawn represents the best fit of the data calculated by a linear regression analysis. The values k_3 and K_i obtained from these data are shown in the lower portion of Table I. The succinyl derivative of Ala-Ala-Pro-Leu-CH_2Cl has a 3-fold lower K_i value than the acetyl derivative and a 75-fold lower K_i value than the free amino derivative. These results suggest that the active site of elastase 2 contains a binding site which can accommodate a negative or neutral residue more readily than a positively charged group. These results are consistent with data obtained on the hydrolysis of peptide *p*-nitroanilides by elastase 2 [9]. In contrast to these

TABLE I

SECOND-ORDER RATE CONSTANTS FOR INHIBITION OF ELASTASE 2 BY SOME PEPTIDE CHLOROMETHYL KETONE DERIVATIVES

Inhibitor	$k_{obs}/[I]$ ($M^{-1} \cdot s^{-1}$)		
Ac-Ala-Ala-Pro-Ala-CH_2Cl	40		
Ac-Ala-Ala-Ala-Ala-CH_2Cl	39		
Ac-Ala-Ala-Pro-Ile -CH_2Cl	4.5		
Ac-Ala-Ala-Pro-Val-CH_2Cl	17		
Suc-Ala-Ala-Pro-Val-CH_2Cl	26		
MeOSuc-Ala-Ala-Pro-Val-CH_2Cl	9		
	k_3 (s^{-1})	$K_i \times 10^6$ (M)	k_3/K_i ($M^{-1} \cdot s^{-1}$)
Suc-Ala-Ala-Pro-Leu-CH_2Cl	0.033	0.74	44 700
Ac-Ala-Ala-Pro-Leu-CH_2Cl	0.040	2.76	14 500
NH_2-Ala-Ala-Pro-Leu-CH_2Cl	0.018	54.7	300

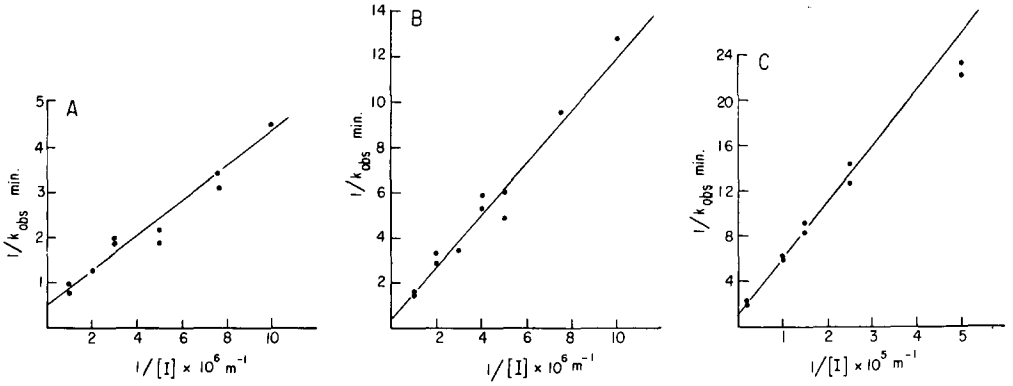

Fig. 1. Inhibition of elastase 2 by X-Ala-Ala-Pro-Leu-CH_2Cl compounds. The dependence of k_{obs} on inhibitor concentration is plotted in accordance with eqn. 3. A. X = succinyl; B. X = acetyl; C. X = H.

differences in K_i, the k_3 values for inhibition of elastase 2 by the three derivatives of X-Ala-Ala-Pro-Leu-CH_2Cl change only 2-fold. This finding confirms the observation of Kettner and Shaw [16] that the k_3 terms did not vary significantly for inhibition of trypsin-like proteases by a series of chloromethyl ketones.

Since $k_{obs}/[I]$ is a good approximation of k_3/K_i when $[I] << K_i$, it is possible to compare the data in Table I in terms of relative reactivity towards elastase 2. Comparison of $k_{obs}/[I]$ and k_3/K_i values for the inhibitors with the structure Ac-Ala-Ala-Pro-amino acid-CH_2Cl shows that the compound containing leucine in the P1 position is the best inhibitor for elastase 2. These data are consistent with the results of studies on the substrate specificity of human elastase 2 which demonstrate that this enzyme preferentially hydrolyzes peptide *p*-nitroanilide derivatives containing leucine in the P1 position [9], and that compounds containing a branch at the β carbon of the P1 amino acid are not good substrates for elastase 2.

Table II presents a comparison of the relative reactivities of peptide chloromethyl ketone inhibitors for several pancreatic proteases. In each case, the inhibitor cited was the best of a series of inhibitors. As can be seen in Table II, human elastase 2 differs from porcine pancreatic elastase 1 and human leukocyte elastase in terms of the P1 amino acid chloromethyl ketone derivative which yields maximal rates of inhibition. Human elastase 2 is preferentially inhibited by compounds containing a P1 leucine while porcine elastase 1 reacts most effectively with alanine compounds [3,4] and leukocyte elastase prefers valine derivatives [5].

The data presented in Table II show that the best inhibitors for human elastase 2 possess second-order rate parameters in the range of $10^4\ M^{-1} \cdot s^{-1}$, similar to those reported for the trypsin inhibitor Phe-Ala-Arg-CH_2Cl [15]. This is in marked contrast to the relatively low kinetic parameters reported for peptide chloromethyl ketone inhibition of bovine chymotrypsin [6], porcine elastase 1 [3,4] and human leukocyte elastase [5]. However, all of these endopeptidases hydrolyze peptide *p*-nitroanilide substrates, the structures of which are analagous to the respective inhibitors, much more rapidly than human

TABLE II

COMPARISON OF KINETIC CONSTANTS FOR INHIBITION OF SERINE PROTEASES BY PEPTIDE CHLOROMETHYL KETONES

Enzyme	Inhibitor	k_{obs}/I ($M^{-1} \cdot s^{-1}$)	k_3/K_i ($M^{-1} \cdot s^{-1}$)	pH	organic solvent
Human elastase 2	Suc-Ala-Ala-Pro-Leu-CH_2Cl	—	44 000	6.85	1% methanol
Porcine pancreatic [a] elastase 1	Ac-Ala-Ala-Pro-Ala-CH_2Cl	38	—	5.0	5% methanol
Porcine pancreatic [b] elastase 2	Ac-Ala-Gly-Phe-CH_2Cl	—	52	8.0	5% dioxane
Human leukocyte [c] elastase	MeO-Suc-Ala-Ala-Pro-Val-CH_2Cl	1 560	—	7.5	4.3% methanol
Bovine chymotrypsin A [d]	Cbz-Gly-Leu-Phe-CH_2Cl	3.23	—	5.8	9% 1,2-dimethoxyethane
Bovine Trypsin [e]	Phe-Ala-Arg-CH_2Cl	50 000	—	7.0	—

[a] Data from Powers and Tuhy [3].
[b] Data from Gertler et al. [14].
[c] Data from Powers et al. [5].
[d] Data from Kurachi et al. [6].
[e] Data from Kettner et al. [15].

elastase 2 hydrolyzes its best substrate [9]. These observations suggest that the kinetic parameters for inhibition of these serine endopeptidases at neutral pH have been significantly underestimated *. It is therefore appropriate to examine the factors which may be responsible for these apparent discrepancies. Firstly, in several previous studies [3,5,6] inhibition reactions were performed at pH 5.0—6.0 in order to decrease the rate of inhibition. However, the pancreatic endopeptidases have bell-shaped pH vs. activity profiles, with maximal activity between approx. pH 7 and 9, and the rate of reaction with substrates falls dramatically below pH 6.5 [17]. The reaction of TPCK with bovine chymotrypsin has been shown to have the same pH dependence [18]. Furthermore, the inhibition of other serine endopeptidases with chloromethyl ketone inhibitors has been very sensitive to small changes in pH [3,5]. Thus the determination of $k_{obs}/[I]$ at pH 5—6 may underestimate the rates of inhibition at neutral pH ranges. In the present study, a very sensitive substrate allowed the use of very low elastase 2 concentrations, so that the rate of inhibition could be determined at a neutral pH value (pH 6.85) while maintaining conditions where a pseudo-first-order kinetic treatment is applicable.

A second factor which may contribute to the low values of $k_{obs}/[I]$ previously reported [3,5,6] is the use of significant and variable amounts of organic solvents in the inhibition reactions. Other investigators have reported that the rate of substrate hydrolysis by serine endopeptidases is highly organic-solvent dependent [19]. Furthermore, Kurachi et al. [6] have demonstrated that $k_{obs}/[I]$ for α-chymotrypsin inhibition by Boc-Ala-Gly-Phe-CH_2Cl at pH 5.8 is 4 times lower in 30% 1,2-dimethoxyethane than that measured in 9% solvent. In the present study, methanol was employed in order to assure complete solubilization of the inhibitors containing N-terminal acetyl groups. The concentration of methanol in the inhibition reaction was kept to a minimum (1%) and was held constant in all experiments. The effect of 1% methanol on the inhibition kinetics was tested by measuring k_{obs} for reaction of elastase 2 with Suc-Ala-Ala-Pro-Leu-CH_2Cl at $2 \cdot 10^{-7}$ M in the presence and absence of 1% methanol. Duplicate determinations indicated that the addition of methanol at this concentration did not change k_{obs}.

A third possible reason for the apparent low values of $k_{obs}/[I]$ is the determination of k_{obs} at inhibitor concentrations which are much greater than K_i. Several previous studies have employed the parameter $k_{obs}/[I]$ to measure inhibition of α-chymotrypsin [6], porcine elastase 1 [3] and leukocyte elastase [5] with inhibitors in the range of $5 \cdot 10^{-5}$ M. In the present study, we have demonstrated a K_i equivalent to $2—5 \cdot 10^{-6}$ M for inhibition of elastase 2 by two peptide chloromethyl ketones with structures similar to those of inhibitors previously employed [3,5] for porcine and leukocyte elastases. Since the K_m value for the hydrolysis of a homologous peptide *p*-nitroanilide substrate by

---

* The second-order rate constant for the inhibition of bovine chymotrypsin by $8 \cdot 10^{-7}$ M Suc-Ala-Ala-Pro-Lue-CH_2Cl at pH 7.4, was determined to be approx. $4 \cdot 10^4$ $M^{-1} \cdot s^{-1}$. The second-order-rate constant for the inhibition of porcine elastase 1 by $4 \cdot 10^{-7}$ M Ac-Ala-Ala-Pro-Ala-CH_2Cl at pH 7.4, was determined to be approx. $6 \cdot 10^4$ $M^{-1} \cdot s^{-1}$. Although the bimolecular rate constant is equivalent to k_3/K_i only when $[I] << K_i$, these values suggest that the rates of inhibition of these proteases by peptide chloromethyl ketones at neutral pH are similar to those for inhibition of elastase 2. (Largman, C., unpublished results.)

porcine elastase 1 (Suc-Ala-Ala-Pro-Ala-pNA) is 10-fold lower than the K_m value for hydrolysis of Suc-Ala-Ala-Pro-Leu-pNA by human elastase 2 [9], it is anticipated that K_i for inhibition of porcine elastase 1 by compounds with the structures listed in Table II should be in the range of 10^{-6}—10^{-7} M. A similar comparison of the K_m values for hydrolysis of peptide substrates by α-chymotrypsin [20] or leukocyte elastase [11] with those of elastase 2 [9] suggests that the best inhibitors for these enzymes should also have K_i values in the range of 10^{-6}—10^{-7} M. Thus the determination of $k_{obs}/[I]$ at inhibitor concentration of 10^{-5} M probably does not fulfill the condition that $[I] << K_i$, at least in the pH range where the enzymes have maximal activity.

The development of efficient tetra-peptide chloromethyl ketone inhibitors for human elastase 2, based on leucine in the P1 position, suggests possible therapeutic use of these compounds in disease states in which active forms of elastase 2 might cause tissue injury. In this regard, we have recently reported the detection of trypsin [21] and elastase 2 (Geokas, M.C., Largman, C. and Brodrick, J.W., unpublished results) bound to α_2-macroglobulin in the plasma of patients with severe pancreatic inflammatory disease. Furthermore, we have demonstrated proteolytic activity in the plasma fraction containing α_2-macroglobulin bound elastase 2 and trypsin in these patients. (The α_2-macroglobulin fraction of plasma from patients with severe pancreatic inflammation rapidly degrades ^{125}I-labeled parathyroid hormone in vitro. Brodrick, J.W., Nissesson, R.A. and Geokas, M.C., unpublished observations.) Future studies in this laboratory will be concerned with the possible therapeutic use of protease inhibitors to reduce the potential degradation of physiologically important polypeptides by circulating forms of pancreatic elastase 2.

References

1 Shaw, E. (1967) Methods Enzymol. 11, 677—686
2 Kettner, C. and Shaw, E. (1978) Biochemistry 22, 4778—4784
3 Powers, J.C. and Tuhy, P.M. (1973) Biochemistry 12, 4767—4773
4 Thompson, R.C. and Blout, E.R. (1973) Biochemistry 12, 44—47
5 Powers, J.C., Grupton, B.F., Harley, A.D., Nishino, N. and Whitley, R.J. (1977) Biochim. Biophys. Acta 485, 156—166
6 Kurachi, K., Powers, J.C. and Wilcox, P.E. (1973) Biochemistry 12, 771—777
7 Stone, P.J., Lucey, E.C., Calore, J.D., Powers, J.C., Snider, G.L. and Franzblau, C. (1979) Am. Rev. Resp. Dis. 119, 364 (Abstract)
8 Largman, C., Brodrick, J.W. and Geokas, M.C. (1976) Biochemistry 15, 2491—2500
9 Del Mar, E.G., Largman, C., Brodrick, J.W., Geokas, M.C., and Fassett, M. (1980) Biochemistry 19, 468—472
10 Kasafirek, E., Frie, P., Slaby, J. and Malis, F. (1976) Eur. J. Biochem. 69, 1—13
11 Nakajima, K., Powers, J.C., Ashe, B.M. and Zimmerman, M. (1979) J. Biol. Chem. 254, 4027—4032
12 Kitz, R. and Wilson, I.B. (1962) J. Biol. Chem. 237, 3245—3249
13 Powers, J.C. (1977) in Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins (Weinstein, B., ed.), Vol. 4, 65—178
14 Gertler, A., Weiss, Y. and Burstein, Y. (1977) Biochemistry 16, 2709—2716
15 Kettner, C., Springhorn, S., Shaw, E., Muller, W. and Fritz, H. (1978) Hoppe-Syeler Z. Physiol. Chem. 359, 1183—1191
16 Kettner, C., and Shaw, E. (1978) Biochemistry 17, 4778—4784
17 Hess, G.P. (1971) Enzymes 3, 213—248
18 Kezdy, F.J., Thomson, A. and Bender, M.L. (1967) J. Am. Chem. Soc. 89, 1004—1009
19 Bieth, J. and Wermuth, C.G. (1973) Biochem. Biophys. Res. Commun. 53, 383—390
20 Del Mar, E.G., Largman, C., Brodrick, J.W. and Geokas, M.C. (1980) Anal. Biochem. 99, 316—320
21 Brodrick, J.W., Geokas, M.C., Largman, C., Fassett, M. and Johnson, J.H. (1979) Am. J. Physiol. 237, E474—E480