The Reaction of Aminoacylase with Chloromethylketone Analogs of Amino Acids

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Aminoacylase, Chloromethyl-Ketone Analogs of Amino Acids, Essential SH and Amino Groups

1. Aminoacylase is irreversibly inactivated by the chloromethylketone analogs of benzyloxycarbonyl-L-alanine, L-alanine, L-leucine, L-aspartic acid (β), tosyl-L-phenylalanine and L-leucyl-L-alanine. The kinetics of the inactivation of the enzyme by the halo-methylketones were investigated.

2. Leucyl- and alanyl chloromethylketone inactivate the enzyme by blocking of 4 SH groups. Experiments with [U-^{14}C]leucyl chloromethylketone confirm that maximal 4 residues are covalently bound to the protein.

3. Inactivation of the enzyme by benzyloxycarbonylalanyl and tosylphenylalanyl chloromethylketone is the result of the substitution of the ε-amino group of one lysine residue per active site and not of SH groups. However, in the presence of competitive inhibitors these halomethylketones react only with the SH groups of the enzyme, too.

In our previous work on aminoacylase we have shown that the enzyme contains two subunits and two active sites with a strong hydrophobic binding center [1–3]. We have further found that the activity of the enzyme depends on at least two SH groups [3] and the presence of two zinc^{2+} ions per molecule [4]. As we have shown by chemical modification, two tryptophan residue are part of the binding center [2] and four histidine molecules are presumably involved in the binding of the metal ions [5]. It was further observed that SH-compounds such as dithiothreitol and mercaptoethanol inactivative the enzyme by splitting of two disulfide bridges, which are obviously essential for the maintainance of the active conformation of aminoacylase [3].

In the present communication we report the results of our experiments on the reaction of chloromethylketone analogs of aminoacids and of a dipeptide with aminoacylase. These experiments give further information on the structure of the active site of the enzyme and suggest that lysine residues contribute to the binding of the substrates and competitive inhibitors.

Materials and Methods

Enzyme

Pig kidney aminoacylase was a gift of Boehringer, Mannheim. The enzyme (spec. activ. about 34 U/mg) was purified by chromatography on Sephadex G 150 and DEAE cellulose up to a spec. activ. of 250 U/mg [3]. The purified enzyme was homogeneous as judged by disc gel and immunoelectrophoresis [3].

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Enzymes: Aminoacylase, N-acylamino acid amidhydrolase, EC 3.5.1.14.

Chemicals

N-Benzyloxycarbonyl-L-alanyl-, L-alanyl-, and L-leucyl-L-alanyl chloromethylketone were synthesized as described by Birch *et al.* [6]. L-Leucyl chloromethylketone was synthesized according to Fittkau [7]. L-Aspartyl-β-chloromethylketone was synthesized according to [8]. N-Tosyl-L-phenylalanyl chloromethylketone (TPCK) was purchased from Merck, Darmstadt. [U-^{14}C]leucin (spec. activ. 5.1 μCi/mM) and [^{14}C]acetic anhydride (spec. activ. 20 μCi/mM) were from Amersham Buchler. N-Chloroacetyl-alanine and N-acetyl-methionine were synthesized according to Greenstein *et al.* [9]. Buffer substances were of p.a. grade from Merck, Darmstadt. Trinitrobenzenesulfonic acid was from Serva, Heidelberg.

Activity measurements

Were performed spectrophotometrically at 236 nm and 238 nm respectively with N-chloroacetyl-alanine and N-acetylmethionine as substrates [1].

Reaction of aminoacylase with chloromethylketones

600 μl enzyme solution (1 mg/ml 0.1 M phosphate/borate buffer, pH 8.0) 40 – 120 μl 5×10^{-3} M chloromethylketone in bidestilled water (TPCK was dissolved in ethanol) and 1360 – 1280 μl 0.1 M phosphate/borate buffer pH 8 were incubated at 40 °C. After 0 – 60 min the reaction was stopped by dilution of 50 μl of the reaction mixture with 1 ml buffer at 0 °C. 5 min later the residual activity was measured by addition of 50 μl N-acetylmethio-

nine to 1 ml of the mixture in a cuvette. The rate constants of inactivation were determined from the time/turnover curves.

pH-Dependence of the inactivation rate of aminoacylase by L-*leucyl*-L-*alanyl chloromethylketone*

40 μl enzyme solution (0.75 mg/ml), 30 μl 5×10^{-3} M leucylalanyl chloromethylketone and 1 ml phosphate/borate buffer pH 7.25 – 8.25 were mixed in a cuvette. After 1, 3, 6 and 10 min 100 μl 25 mM N-chloro-acetylalanine were added to each sample to determine the remaining activity.

Determination of the inhibitor constants

40 – 300 μl 2.5×10^{-2} M N-chloroacetylalanine and 20 – 300 μl 10^{-2} M chloromethylketone were mixed in a cuvette with 920 – 380 μl 0.1 M phosphate/borate buffer pH 8.0. The reaction was started by 20 μl enzyme solution (0.75 mg/ml); the hydrolysis of the substrate was followed for two minutes. The inhibitor constants were determined from a Dixon plot [10].

Effect of the halomethylketones on the SH groups of the enzyme

1.5 mg aminoacylase in 1 ml buffer containing 10^{-2} M chloromethylketone were incubated at 40 °C in a cuvette for 1 and 2 hours respectively. The remaining activity was determined with N-chloroacetyl-alanine. 2 ml performic acid were added; after 4 hours at 0 °C the samples were dialysed against bidestilled water for 16 hours at 4 °C. Then the enzyme was hydrolyzed in 6 N HCl for 22 hours at 110 °C. The number of cysteic acid residues was determined with the amino acid analyzer Beckmann Multichron B. The number of substituted SH groups was calculated from the difference to the control values.

The effect of the competitive inhibitors N-tosyl-alanine and DL-norleucine on the modification of the SH groups of aminoacylase by benzyloxycarbonyl-L-alanyl chloromethylketone and L-alanyl chloromethylketone was studied by incubation of 1 ml enzyme solution (1.2 mg/ml) with 200 μl 5×10^{-3} chloromethylketone in the presence and absence of 200 μl 2×10^{-3} M N-tosylalanine and 4×10^{-3} M DL-norleucine respectively. The samples were reacted for 1 hour at 40 °C and the residual activity was determined with N-chloroacetyl-alanine. The extent of modification of the SH groups was determined as described above.

Covalent labelling of the enzyme with [U-^{14}C]leucyl-chloromethylketone

Samples of the enzyme were incubated with [U-^{14}C]leucyl-chloromethylketone as described above. The reaction was stopped after appropriate time intervals by dilution with cold 1 M acetate buffer pH 5.0 and dialysed against bidestilled water. Samples were counted in a Packard Tricarb 3003 after mixing with 10 ml Aquasol. The number of residues incorporated was calculated on the basis of the spec. activity of the labelled halomethylketone.

Modification of lysine residues

Acetylation with acetic anhydride

The enzyme solution (1 mg/ml) was dialysed against a 0.2 M phosphate/borate buffer pH 6.5, pH 7.5 or pH 8.5. The enzyme was then reacted with a 30 – 8000 fold excess of [^{14}C]acetic anhydride until the reagent was completely decomposed. The reaction mixture was dialysed against water to constant radioactivity. Aliquots of 500 μl were mixed with 15 ml Aquasol and were counted in a Tricarb. The extent of modification of amino groups was calculated from the protein concentration and the spec. activity of the acetic anhydride.

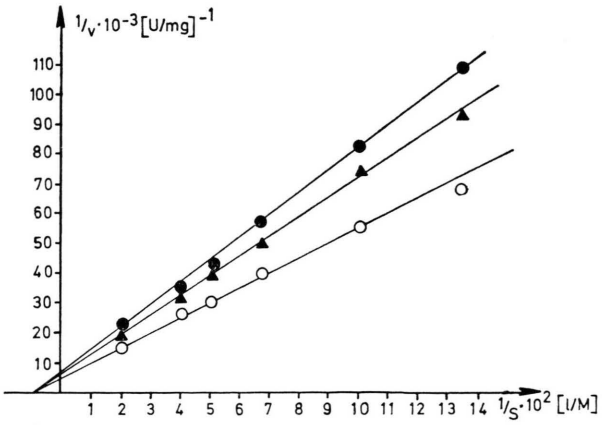
Reaction with trinitrobenzene sulfonic acid (TNBS)

The enzyme solution (1 mg/ml) was dialyzed overnight at 4 °C against a 0.2 M carbonate buffer pH 8.5. 500 μl of the solution were mixed with 100 μl 0.1% TNBS solution at 40 °C. After appropriate time intervals the reaction was stopped by addition of 100 μl 0.2 M Tris buffer pH 8.0 and cooling to 0 °C. The remaining activity was measured. The number of modified amino groups was determined photometrically at 345 nm as described by Habeeb [11] or by amino acid analysis after reduction of the trinitroanilin derivatives with 0.2 M Sodiumdithionite for 50 min at 40 °C and hydrolysis of the protein in the usual manner. Aminoacid analysis reveales the loss of lysine residues.

Results

Kinetics of the inactivation of aminoacylase by halomethylketones

Halomethylketone analogs of protected and unprotected amino acids and dipeptides are potent inhibitors of aminoacylase. The kinetics of the inhibition were studied in the presence of various concentrations of chloromethylketones. Lineweaver-Burk graphs [12] were plotted to evaluate the type of inhibition. Typical Lineweaver-Burk plots are shown in Fig. 1. An uncompetitive type of inhibition was observed with all halomethylketones tested. The inhibitor constants were determined from the corresponding Dixon plots $1/V$ versus $[J]$. Examples are given in Fig. 2. The K_i values of the various



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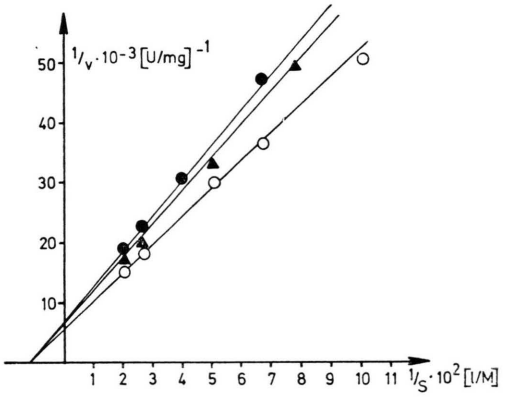

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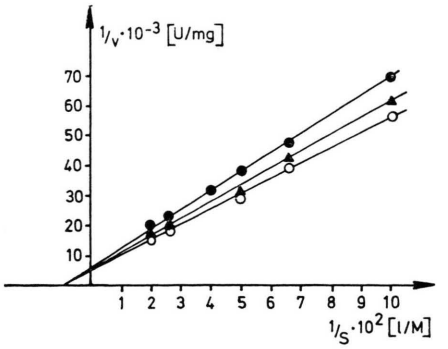

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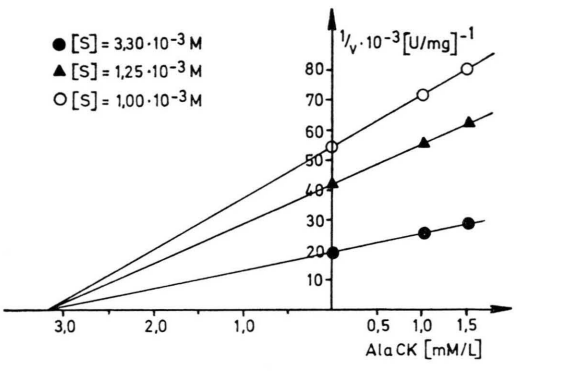
Fig. 1. Lineweaver Burk plots of the inhibition of aminoacylase by halomethylketones. Substrate: Chloroacetyl alanine;

a) ○—○ without inhibitor;
▲—▲ 1.0×10^{-3} M alanyl chloromethylketone;
●—● 1.5×10^{-3} M alanyl chloromethylketone;
b) ○—○ without inhibitor;
▲—▲ 2×10^{-4} M leucyl-alanyl chloromethylketone;
●—● 3×10^{-4} M leucyl-alanyl chloromethylketone;
c) ○—○ without inhibitor;
▲—▲ 5×10^{-5} M tosylphenylalanyl chloromethylketone;
●—● 10^{-4} M tosylphenylalanyl chloromethylketone.



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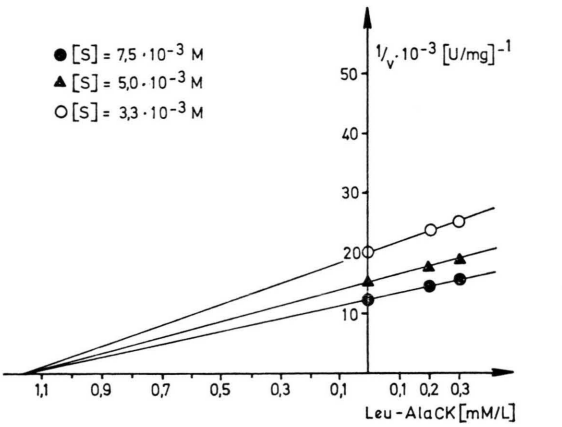

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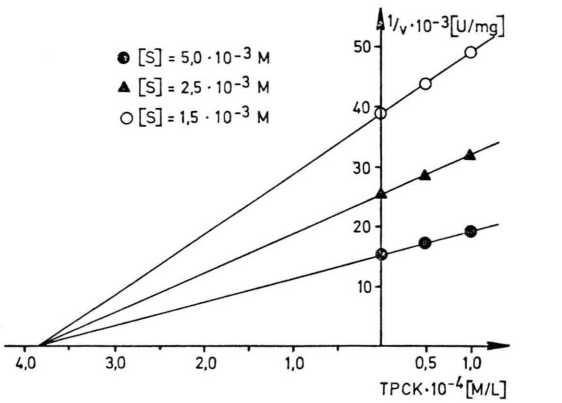


c)

Fig. 2. Dixon plots of the inhibition of aminoacylase by
a) alanyl chloromethylketone;
b) leucyl-alanyl chloromethylketone;
c) tosylphenylalanyl chloromethylketone.

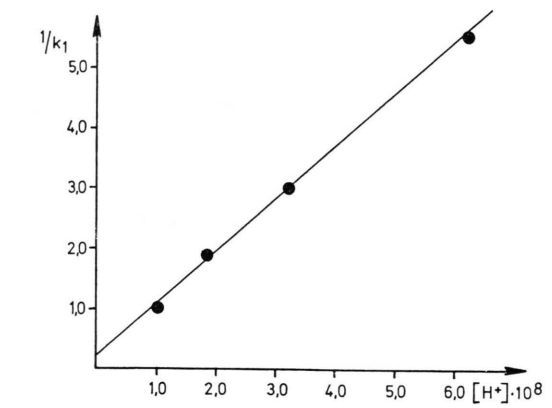
Table I. Inhibitor constants of the noncompetitive inhibition of aminoacylase by various chloromethylketones and second order rate constants of inactivation at pH 8.0.

Chloromethyl-ketone	$K_i \times 10^3$ [M/l]	Affinity $[1/K_i]$	rel. Aff.	$k_2 \times 10^{-3}$ $[l \times mol^{-1} \times sec^{-1}]$
Alanyl	3.17	0.31	11.7	1.97
Leucyl	7.70	0.13	5.0	1.46
Leucyl-alanyl	1.15	0.87	36.0	1.20
Tosylphenyl-alanyl	0.38	2.63	100	0.91
β-Aspartyl	1.37	0.73	27.6	0.13

chloromethylketones obtained from the Dixon plots are summarized in Table I.

In order to obtain some general information on the sensitivity of aminoacylase against halomethylketones of different structure, we have measured the rate of inactivation of the enzyme by the chloromethylketones. The second order rate constants of inactivation of the enzyme are also shown in Table I. This rate constants describe the rate of covalent inactivation by reaction of a functional group of the active site or nearby with the chloromethylketones. From a comparison of the constants of Table I the following conclusions may be drawn: There is no simple correlation between the rate of inactivation and the inhibitor constants, which are a measure of the affinity between the enzyme and the inhibitor. The highest affinity is observed for TPCK, the lowest value is found for leucyl-chloromethylketone, the affinity of which is twenty times lower than that of TPCK. With the inhibitor of the highest affinity surprisingly the lowest rate of inactivation was measured *. The affinity of the most reactive alanyl chloromethylketone is only 10% of the affinity of the compound with the highest affinity. From these results we must conclude that the reaction partners on the protein of the various types

* β-Aspartyl-chloromethylketone is a special case.



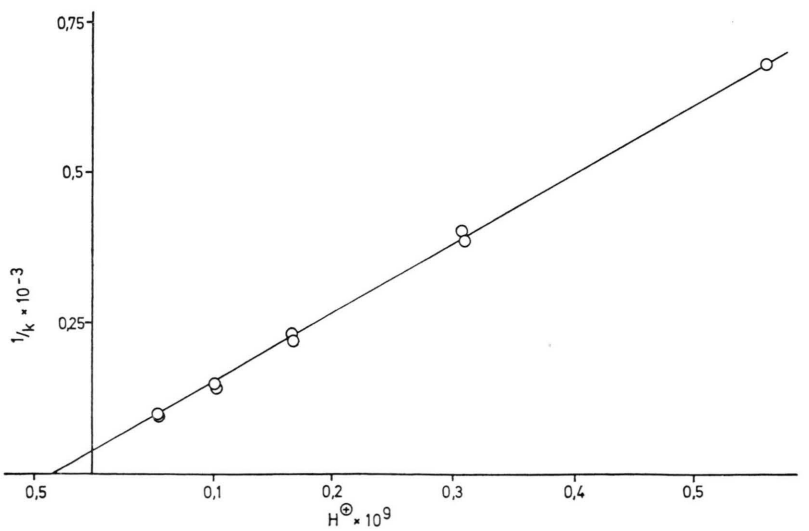


Fig. 3.

a) pH dependence of the rate of inactivation of aminoacylase by leucyl-alanyl chloromethylketone, "Lindley diagram". The pK value was calculated from the Lindley equation [13];

b) pH dependence of the rate of inactivation of aminoacylase by tosylphenylalanyl chloromethylketone. The pK value was calculated from the Lindley equation [13].

of chloromethylketones are different. This assumption was confirmed by studies on the pH dependence of the rate of inactivation and the identification of the modified residues.

pH Dependence of the rate of inactivation of aminoacylase by leucyl-alanyl and tosylphenylalanyl-chloromethylketone

To obtain first indications on the functional groups of the protein which react with the halomethylketones, the pH dependence of the rate of inactivation of aminoacylase by leucylalanyl and tosylphenylalanyl chloromethylketone was studied. Surprisingly two significantly different pK values were obtained. The pH dependence of the inactivation rate of the enzyme by the dipeptide chloromethylketone is characterized by a pK of about 8.3 as evaluated from a Lindley diagram $1/k_1$ versus $[H^+]$ [13] (see Fig. 3 a). This value points out to a normal pK of the SH group of a cysteine residue. Our further experiments confirm this assumption.

However, the pH-dependence of the inactivation of aminoacylase by tosylphenylalanyl chloromethylketone yields a pK of 10.4 as evaluated from a Lindley diagram, too (see Fig. 3 b). This value draws attention to an ε-amino group of lysine.

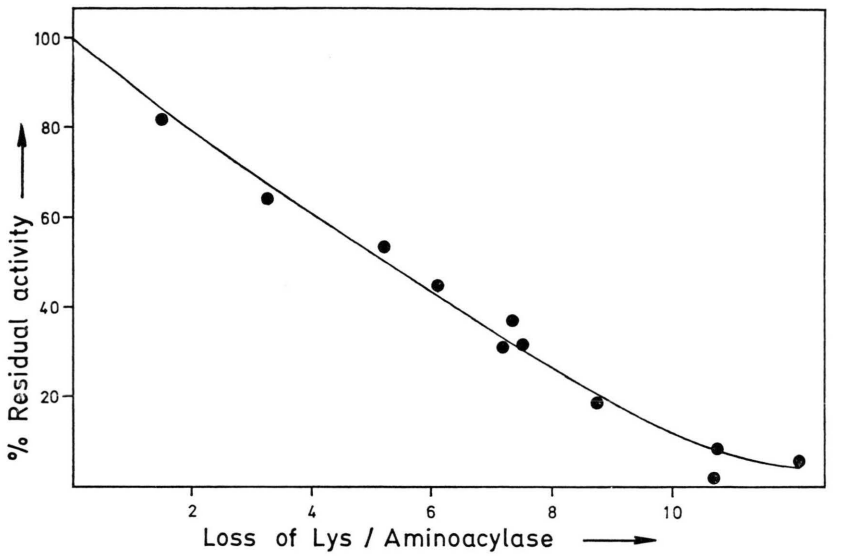


Fig. 4. Relation between residual activity and the number of modified lysine residues by trinitrobenzenesulfonic acid. Loss of lysine was determined by amino acid analysis after reduction of the trinitroaniline residues with dithionite.

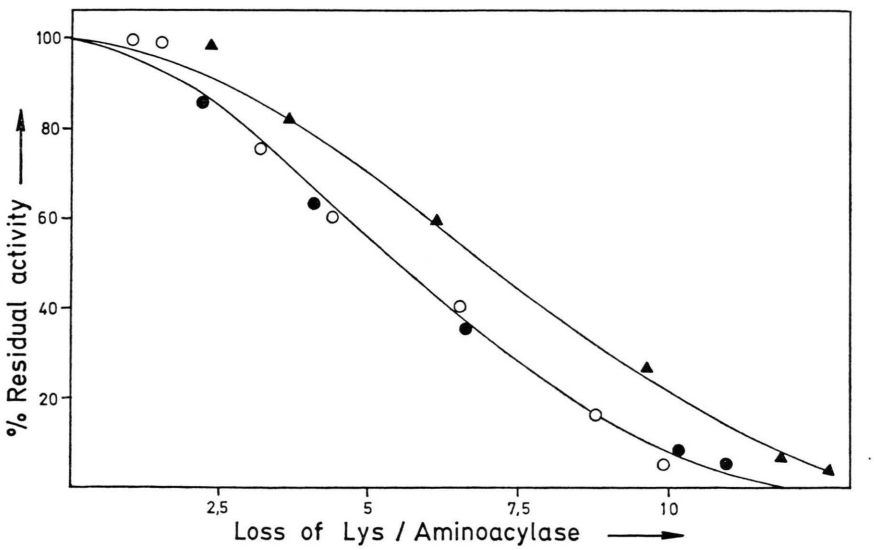


Fig. 5. Relation between residual activity and number of modified lysine residues by [^{14}C]acetic anhydrid at pH 8.5 ▲—▲, pH 7.5 ●—●, pH 6.5 ○—○.

Chemical modification of the amino groups of amino acylase

In order to evaluate the function of the amino groups of the enzyme, the effect of chemical modification of the lysine residues on the catalytic activity was studied. The relation between the degree of lysine modification and remaining activity after reaction of the enzyme with trinitrobenzenesulfonic acid or acetic anhydride is shown in Figs 4 and 5. In both cases a nearly total loss of activity is observed only after modification of about 10 – 12 lysine residues out of 34 present in the enzyme. This loss of activity is not a consequence of the dissociation of the enzyme into subunits as was shown by immuno electrophoresis. Obviously it is not possible with this reagents to detect specifically only essential amino groups of the active site region, because the enzyme containes 34 lysine residues most of which are on the surface of the protein molecule and are easily available for the modification reagents. However, we may conclude from these experiments that amino groups have something to do with the activity of aminoacylase.

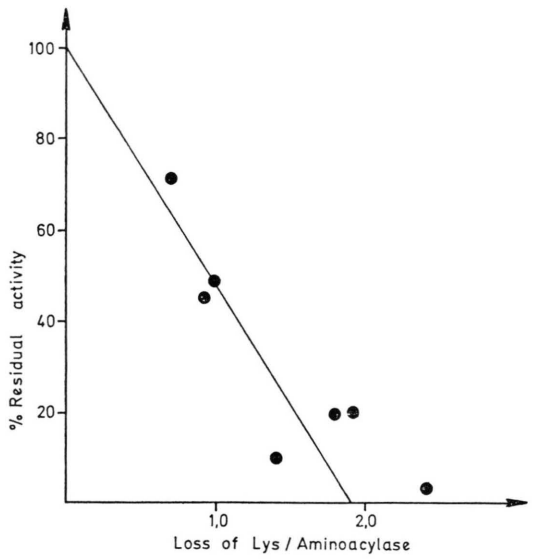


Fig. 6. Relation between residual activity and number of modified lysine residues after incubation with tosylphenylalanine chloromethylketone. The loss of lysine residues was determined by amino acid analysis after reduction of the modified protein with sodium borohydride.

Further indications on the possible function of amino groups came from the inactivation experiments with tosylphenylalanine chloromethylketone (TPCK). The relation between the remaining activity and the loss of lysine residues after incubation with TPCK is illustrated in Fig. 6. From this figure it becomes likely that inactivation of the enzyme takes place after substitution of two lysine residues by TPCK. It is important to mention that even on prolonged incubation of amino acylase with TPCK the loss of lysine does not significantly exceed two residues. The protective effect of the competitive inhibitor DL-norleucine on the inactivation of the enzyme by TPCK is shown in Fig. 7. A concentration of 6×10^{-4} M DL-norleucine nearly completely prevents the inactivation of aminoacylase.

Direct proof of ε-amino groups of lysine residues being the point of alkylation by TPCK came from performic acid oxidation [14] experiments of the enzyme after irreversible inactivation with TPCK. As was shown by Schoellmann and coworkers [15], performic acid oxidation of residues alkylated by chloromethyl ketones results in the formation of the corresponding carboxymethyl derivatives of the attacked amino acid residue. In our experiments N^{ε}-carboxymethyllysine could be detected by amino acid analysis (N^{ε}-carboxymethyl lysine appears shortly after changing the buffer before methionine) in a hydrolysate of the enzyme after inactivation with TPCK, indicating alkylation at the ε-amino groups of lysine residues of the binding site. It must be emphasized that TPCK does not react with the ε-amino groups of "normal" lysine residues of proteins. Since no stochiometric oxidation takes place it is difficult to quantify the degree of modification of ε-amino groups by TPCK in this way. But under *no circumstances* more than about two N^{ε}-carboxymethyllysine residues could be detected.

Incorporation of [U-^{14}C]leucyl chloromethylketone

In order to elucidate the number of chloromethylketone residues incorporated into the enzyme [U-^{14}C]leucyl chloromethylketone was reacted with aminoacylase. The amount of radioactivity irreversibly bound to the protein allowes the calculation of the number of substituted residues. The relation between residual activity and the number of leucyl chloromethyl residues incorporated is shown in Fig. 8. From this figure one recognizes that complete inactivation of the enzyme is observed after incorporation of 3 – 4 residues. The question must now be answered as to the nature of the amino-acids modified.

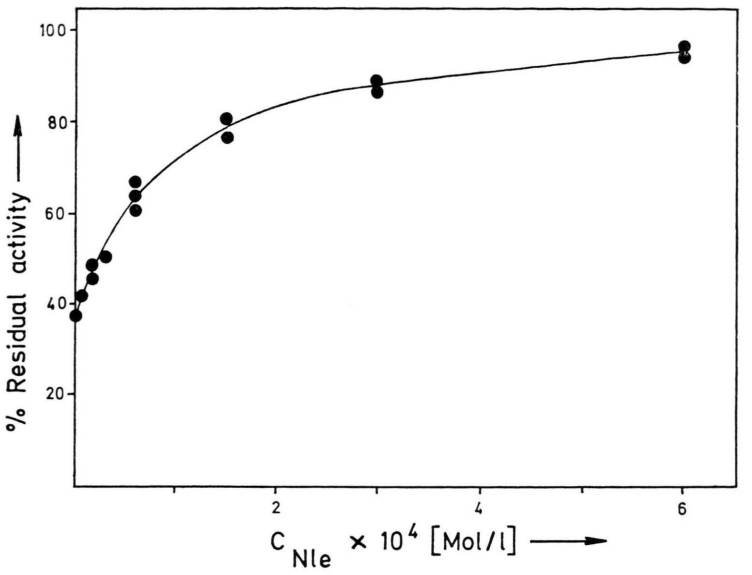


Fig. 7. Protective effect of the competitive inhibitor DL norleucine on the inactivation of aminoacylase by tosylphenylalanine chloromethylketone. The enzyme was incubated 10 min at 40 °C with the chloromethylketone.

Effect of leucyl-, alanyl-, benzyloxycarbonylalanyl- and tosylphenylalanyl chloromethylketone on the SH groups of aminoacylase

As we have shown in a previous communication [3], aminoacylase contains four SH groups which seem to be parts of the active sites of the enzyme. Chemical modification of two of these SH groups abolishes the catalytic activity. Consequently we have looked for the effect of the chloromethylketones on the SH groups, in as much as the pH dependence of the inactivation by leucyl-alanyl chloromethylketone draw attention to the thiol groups (see above). The extent of modification of the SH groups was evaluated with the aminoacid analyzer after performic acid oxidation. The results of these experiments are summarized in Table II.

Table II. Modification of the SH groups of aminoacylase by chloromethylketones.

Chloromethylketone	Incubation time	Inactivation [%]	Number of modified SH groups
Alanyl-	1	97.3	3.8
Leucyl-	2	100.0	4.0
Z *-Alanyl-	1	70.7	0.1
Tos **-phenylalanyl-	2	100.0	1.0
Z *-Alanyl- +Tos **-alanin	1	78.3	3.3
Z *-Alanyl- +DL-norleucine	1	82.2	3.7

* Z=benzyloxycarbonyl-, ** Tos=tosyl.

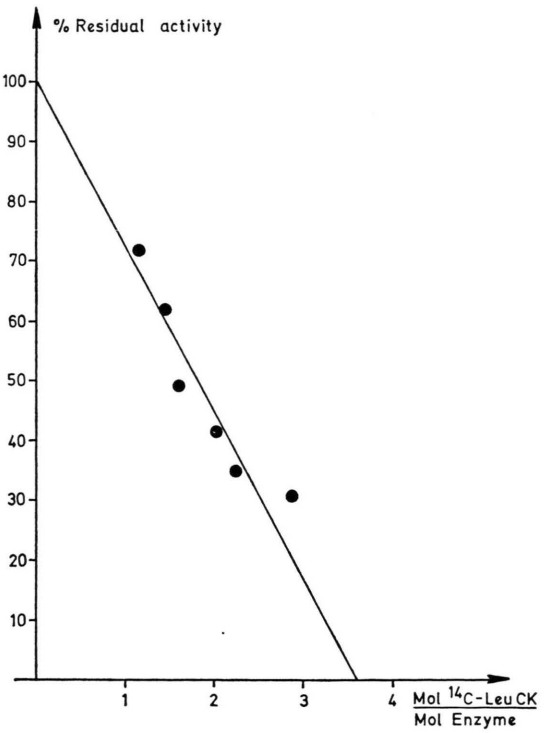


Fig. 8. Relation between residual activity and number of [^{14}C]leucyl chloromethylketone residues irreversibly incorporated into aminoacylase after incubation between 5 and 60 min.

The following conclusions may be drawn from these experiments: Complete inactivation of the enzyme is observed after modification of 4 SH

groups. This value is in fairly good agreement with the results of the incorporation experiments described above for leucyl-chloromethylketone. In contrast complete inactivation by tosylphenylalanyl chloromethylketone was found after modification of only 1 SH group; further the number of modified thiol groups does not rise if the inactivation increases from 30% to 100%. However, in the case of leucylchloromethylketone a correlation between the inactivation and the number of modified sulfhydryl groups is observed. We therefore must conclude that tosylphenylalanine chloromethylketone attacks additional essential aminoacids at the active site of aminoacylase. The pH-dependence of the inactivation by TPCK and the loss of lysine residues observed (see above) suggest that this are lysine residues. We assume that the ε-aminogroups of lysines in the cationic state are important for the binding of the substrates and competitive inhibitors by electrostatic interaction with their anionic carboxyl groups. This idea is supported by the observation that acylaminoacids and dipeptides with blocked carboxyl groups (esters or amides) are not accepted as substrates at all and are much weaker competitive inhibitors of the enzyme as compared to the corresponding compounds with free carboxyl groups (anionic site). For instance K_i of leucine is 0.48 mM, of leucineamide 21 mM and leucinemethylester 18 mM. Further informations on the possible arrangement of the ε-amino and SH groups in the active center came from experiments with benzyloxycarbonylalanyl chloromethylketone and the effect of competitive inhibitors on the modification reaction by this halomethylketone. As we have already described, alanyl chloromethylketone reacts like the leucyl compound with SH groups. However, benzyloxycarbonylalanyl chloromethylketone reacts more like tosylphenylalanyl chloromethylketone: SH groups are not all attacked by this reagent as is shown by amino acid analysis; but no SH groups are accessible with Ellman reagent after covalent labeling with this halomethylketone. The high degree of inactivation of the enzyme by benzyloxycarbonylalanylchlormethylketone must therefore be the result of modification of other residue of the enzyme, namely of ε-amino groups. The picture changes completely if the reaction with this halomethylketone is performed in the presence of the competitive inhibitors DL-norleucine and tosylalanine. Now the extent of reaction of the SH groups with benzyloxycarbonylalanyl chloromethylketone approaches nearly 4. Consequently competitive inhibitors favour the reaction of substrate like halomethylketones as benzyloxycarbonylalanyl chloromethylketone with the SH groups.

Concerning the structure of the active site of amino acylase we may draw the following conclusions from the present results: A free carboxyl group (anionic site) is essential for the optimal binding of substrates (acylaminoacids, dipeptides) and competitive inhibitors. This anionic site interacts with a cationic site presumably an ε-aminogroup of lysine in the protonated state. Those halomethylketones which mostly resemble the substrate attack preferentially the essential amino groups of the active centers; this are for instance tosylphenylalanyl- and benzyloxycarbonyl-alanyl chloromethylketone which exhibit the highest affinity to the enzyme. Hydrophobic interactions with the active site are preferentially responsible for the binding of this compounds. The chloromethylketone analogs of free aminoacids attack in a "less specific" reaction the SH groups of the enzyme. Competitive inhibitors with a free carboxyl group protect the ε-amino group because they occupy a part of the binding center. They also reduce the rate of reaction of the chloromethylketone analogs of free aminoacids with the SH groups which are part of the catalytic center, too.

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[1] W. Kördel, Thesis, University of Marburg 1976.
[2] W. Kördel and Fr. Schneider, Hoppe-Seyler's Z. Physiolog. Chem. **357,** 1109–1115 [1976].
[3] W. Kördel and Fr. Schneider, Biochim. Biophys. Acta **445,** 446–447 [1976].
[4] W. Kördel and Fr. Schneider, Z. Naturforsch. **32 c,** 337–341 [1977].
[5] W. Kördel and Fr. Schneider, Z. Naturforsch. **32 c,** 342–344 [1977].
[6] P. L. Birck, H. A. El-Obcid, and M. Akktar, Arch. Biochem. Biophys. **148,** 447–451 [1972].
[7] S. Fittkan, J. Prakt. Chem. **315,** 1037 [1973].
[8] E. Khadouri, P. M. Anderson, and A. Meister, Biochemistry **5,** 3552–3557 [1966].
[9] S. M. Birnbaum, L. Lernitow, R. B. Kingsley, and J. P. Greenstein, J. Biol. Chem. **194,** 455–470 [1952].
[10] M. Dixon, Biochem. J. **55,** 170–174 [1953].
[11] A. F. S. A. Habeeb, Anal. Biochem. **14,** 328–336 [1966].
[12] H. Lineweaver and D. J. Burk, J. Am. Chem. Soc. **56,** 658–663 [1934].
[13] H. Lindley, Biochem. J. **82,** 418–425 [1966].
[14] C. H. W. Hirs, J. Biol. Chem. **219,** 611–616 [1956].
[15] E. B. Ong, A. J. Johnson, and G. Schoellmann, Biochim. Biophys. Acta **429** (1), 252–257 [1976].