Biochimica et Biophysica Acta, 445 (1976) 446—457

BBA 67888

CHEMICAL INVESTIGATIONS ON PIG KIDNEY AMINOACYLASE

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(Received March 1st, 1976)

Summary

1. Preparations of purified pig kidney aminoacylase (*N*-Acylamino-acid amidohydrolase, EC 3.5.1.14) were obtained by Sephadex and DEAE-cellulose chromatography in homogeneous form as judged by polyacrylamide gel electrophoresis and immunoelectrophoresis.

2. The apparent molecular weight of the enzyme, determined by gel filtration, was about 86 000. After treatment with mercaptoethanol, performic acid or sodium dodecyl sulphate a band with an apparent molecular weight of approximately 43 000 was observed in polyacrylamide gels containing sodium dodecyl sulphate. Thus pig kidney aminoacylase seems to be composed of two subunits.

3. The amino acid composition of the enzyme was determined. Aminoacylase contains 772 amino acids, which corresponds to a molecular weight of 85 500. 12 tryptophan and 12 half-cystine residues were found.

4. Each subunit of the enzyme contains two -SH groups of different reactivity and two disulfide bonds one of which is easily cleaved by -SH compounds, the second only by performic acid oxidation.

5. Chemical modification of two -SH groups abolishes the catalytic activity of aminoacylase. Cleavage of two disulfide bonds also inactivates the enzyme. It is suggested that the enzyme has two active sites each containing an essential -SH group and disulfide bond. One active site is assumed to be part of each subunit.

Aminoacylase (*N*-Acylamino-acid amidohydrolase, EC 3.5.1.14) catalyses the hydrolysis of *N*-acylamino-acids [1] and, at somewhat higher pH values, of

Abbreviations: Ellman reagent, 5,5′-Dithiobis(2-nitrobenzoic acid); Tris, Tris(hydroxymethyl)-amino-methane; SDS, Sodium dodecyl sulphate; Tos-Phe-CH_2Cl, L-1-tosylamido-L-phenyl-ethyl-chloromethylketone.

dipeptide substrates [2]. The enzyme exhibits maximal activity with substrates containing hydrophobic amino acids [2]. Though aminoacylase was first described in 1881 by Schmiedeberg [3] little is known about the structural properties and the mechanism of action of the enzyme. Only two papers have been written [4,5] on the purification and selected physicochemical properties of aminoacylase. Some kinetic experiments on the mechanism of aminoacylase were published by Morawcsick et al. [6] in 1971.

In the present communication we continue our reports [2,7] on the investigation of the structural properties and the catalytic mechanism of this enzyme. The topics of this work are the amino acid composition, the cysteine and cystine content, the subunit structure and the reactivity and function of the -SH groups of the enzyme.

Materials and Methods

Pig kidney aminoacylase was a gift of Boehringer, Mannheim. Purification of the enzyme is described below. *N*-chloroacetyl-L-alanine was synthesized according to the method of Greenstein et al. [8]. Thioglycolic acid, TosPheCH$_2$-Cl, 1,3-dichloro-2-propanone, chloroacetonitrile, 2-hydroxy-5-nitrobenzylbromide, vinylpyridine and buffer reagents were from Merck, Darmstadt. Reference proteins for molecular weight determinations were from Serva, Heidelberg or Boehringer, Mannheim. Asparaginase was a gift of Bayer.

Activity measurements were performed spectrophotometrically with a Zeiss PMQ II or Beckman Acta III spectrophotometer. Hydrolysis of *N*-chloroacetyl-L-alanine was followed at 238 nm. Sephadex G-150 and G-25 were from Pharmacia, Frankfurt, DEAE-cellulose was from Serva, Heidelberg.

Purification of the enzyme

The starting material was a suspension of pig kidney aminoacylase in ammonium sulphate solution with a specific activity of about 34 units/mg protein. The protein was centrifuged and 2 g of precipitate dissolved in 100 ml 10 mM Tris buffer pH 7.3. The solution was passed through a Sephadex G-150 column (10 × 100 cm) equilibrated with the same buffer. To avoid interference from residual ammonium sulphate, an upward flow was used with a flow rate of 100 ml per h; 25-ml fractions were collected. The active fractions (about 1000 ml) were combined and applied to a DEAE-cellulose column (2 × 15 cm) equilibrated with the 10 mM Tris buffer pH 7.3. The flow rate was 40 ml per h. After equilibration of the column with 200 ml of 50 mM Tris buffer, pH 7.3, the protein was eluted with a linear gradient in the range 50—200 mM Tris buffer, pH 7.3, collecting 5-ml fractions. Protein concentration and activity of the fractions were assayed spectrophotometrically. The active fractions were combined and stored in a frozen state (protein content between 1 and 3 mg/ml). Lyophilization of the enzyme leads to appreciable loss of activity.

Molecular weight determinations

Molecular weight determinations by SDS polyacrylamide gel electrophoresis were performed according to the method of Weber and Osborn [9]. Aminoacylase and reference proteins were incubated at 37°C for 2 h in 0.01 M sodi-

um phosphate buffer pH 7.0, 1% in SDS and 1% in 2-mercaptoethanol. A 7.5% polyacrylamide gel was used.

Further electrophoretic studies were carried out with the same proteins after performic acid oxidation as described by Hirs [10]. Samples of the proteins were oxidized in 2 ml performic acid for 5 h at 0° C. The oxidation mixture was diluted to 10 ml with water and lyophilized. The samples were dissolved in buffer and subjected to electrophoresis.

For molecular weight determinations of the polypeptide chains of aminoacylase the electrophoretic mobilities were plotted against the logarithms of the known polypeptide chain molecular weights of the reference proteins as described in ref. 9.

Additional molecular weight determinations were carried out by chromatography on a Sephadex G-150 column [11]. In these experiments reference proteins were carbonic anhydrase, hemoglobin, alkaline phosphatase, catalase, asparaginase and ovalbumin. For the determination of the specific molar extinction coefficient the enzyme was dialyzed against 0.1 M carbonate buffer pH 8.5. The extinction of the solution at 280 nm was measured and the protein content of 1 ml was determined by amino acid analysis. $E_{1\ cm}$ at 280 nm was found to be 13.5.

Amino acid analysis

For amino acid analysis a Beckman Multichrom B Analyser was used. Samples of aminoacylase solution in buffer (1—3 mg/ml) were dialysed against water and diluted with 10 M HCl to a final concentration of 6 M. The solutions were freed from oxygen by bubbling argon through the samples before sealing. After 22 h at 110° C the samples were evaporated at 40° C and dissolved in application buffer. For tryptophan determinations the hydrolysis was performed according to the method of Matsubara and Sasaki [12] in the presence of 5% thioglycolic acid. For further details see ref. 12. Methionine was also determined under protection with thioglycolic acid and thiodiglycol (25 ml in 5 l buffer). After performic acid oxidation it was determined as methioninesulfone.

Cysteine and cystine were determined as cysteic acid after performic acid oxidation. To provide controls for the reliability of the determination, the enzymes asparaginase and papain as well as a mixture of cysteine, glutamic acid, alanine and glycine were oxidized and hydrolyzed under the same conditions.

To test the availability of the -SH groups of aminoacylase the enzyme was incubated for one hour with $5 \cdot 10^{-2}$ M vinylpyridine [13] at pH 8.0. The excess reagent was removed by dialysis. The number of substituted cysteine residues was either determined with the amino acid analyzer using synthetic *S*-β[4-pyridylethyl]-cysteine as a standard or after performic acid oxidation of the modified protein using the difference in the cysteic acid content between the controls and the vinylpyridine-treated enzyme. The reference sample of *S*-β[4-pyridylethyl]-cysteine was synthesized by reaction of cysteine with an excess of vinylpyridine. *S*-β[4-pyridylethyl]-cysteine was determined on the short column of the amino acid analyzer using Beckman M-72 resin. The compound appeared after arginine with the pH 4.95 elution buffer.

Inhibition experiments

Inhibitory effects of metal ions (Zn^{2+}, Cd^{2+}, Hg^{2+}, Co^{2+}, Mg^{2+}) on the activity of aminoacylase were tested by the following procedure: The hydrolysis of *N*-chloroacetyl-L-alanine by aminoacylase was followed in 0.1 M phosphate/borate buffer pH 8.0 and 40°C for 1 min. 5—50 µl of 2×10^{-3}—2×10^{-2} M metal salt solutions were added, mixed and the reaction measured for further 5 min.

The effects of disulfide reducing agents on the activity of aminoacylase were measured by incubation of the enzyme in 0.1 M phosphate/borate buffer pH 8.0 for 10 min at 40°C with the reducing agent (dithiothreitol, mercaptoethanol, cysteine, sodium hydrogen-sulfide) in a concentration of $5 \cdot 10^{-5}$—$5 \cdot 10^{-3}$ M. Thereafter the activity was measured by addition of *N*-chloroacetyl-L-alanine; the reaction was carried out in a cuvette.

For the measurement of the rate of inactivation of aminoacylase by the alkylating agents iodoacetamide, 1,3-dichloro-2-propanone, chloroacetonitrile, 2-hydroxy-5-nitrobenzylbromide and vinylpyridine the enzyme was dialyzed against 0.1 M Tris buffer, pH 8.0. The enzyme solution (0.5 ml, 2 mg/ml) was incubated with the reagent (final concentration 5 mM) at 25°C. At different time intervals, 20 µl of the incubation mixture were transferred into a prepared cuvette and the activity was measured in the usual manner.

The reaction of aminoacylase with Ellman reagent [14] and carboxypyridine disulfide [15] was studied in the presence and absence of competitive inhibitors (norleucine and *N*-tosyl-L-alanine). Enzyme solutions (1 ml, 2 mg/ml) in Tris buffer (pH 8.0) were mixed with 1 ml of the same buffer or 1 ml of the competitive inhibitor (20 mM D,L-norleucine or 50 mM *N*-tosyl-L-alanine). The reaction was started with 100 µl 5 mM Ellman reagent or carboxypyridine disulfide and followed spectrophotometrically at 412 nm or 344 nm respectively. The p*K* values of the -SH groups were evaluated from the pH dependence of the reaction rate with *N*-ethylmaleimide. The enzyme (2 mg/ml) was dialyzed against 10 mM Tris buffer pH 8.5 for 4 h. 50 µl of aminoacylase were diluted to 500 µl with a 0.1 M Tris buffer of the appropriate pH. The reaction was started by addition of 20 µl *N*-ethylmaleimide solution in water (final concentration $1.5 \cdot 10^{-4}$ M) at 0°C. After time intervals ranging from 0.5 to 10 min, the reaction was stopped by addition of 20 µl 10 mM cysteine and 500 µl cold 0.2 M sodium acetate buffer pH 5.0. The low-molecular-weight compounds were separated on a Sephadex G-25 column; the protein concentrations and residual activities were measured as described.

Results and Discussion

Purification of the enzyme

The starting material for our purification was an enzyme preparation of pig kidney aminoacylase having a specific activity of 34 units. A homogeneous enzyme was obtained from this raw material by gel filtration on Sephadex G-150 and DEAE cellulose chromatography. Typical results of the enzyme purification are presented in Table I. The described procedure makes it possible to obtain a 40% yield and a 7.3-fold purification.

When subjected to polyacrylamide gel electrophoresis 300 µg purified enzyme shows a single band on protein staining.

TABLE I

PURIFICATION OF PIG KIDNEY AMINOACYLASE STARTING WITH A COMMERCIAL PREPARATION

Step	Protein (mg)	Activity (units)	Specific activity (units)	Purification	Recovery of activity
Commercial preparation	2000	70 000	34	—	100
Sephadex G-150 eluate	550	38 500	68	2	55
DEAE-cellulose eluate	112	28 000	250	7.3	40

It was however not possible to obtain a single sharp band in the electrophoresis (see below). The purity of the enzyme was therefore tested also by immunoelectrophoresis. With this technique only a single sharp band was obtained (Löffler, H.G., unpublished results). It was not possible to purify aminoacylase by preparative gel electrophoresis because the activity was nearly completely lost. Obviously the enzyme is denatured during gel electrophoresis. This seems also to be the reason for the diffuse bands after protein staining. The isoelectric point was found to be 5.0. The specific activity of the enzyme preparation of Bruns and Schulze [5] was 120 units/mg protein at pH 7.0; with our product we found 250 units/mg protein at pH 7.8. Since the activity at pH 7.0 is about 60% of that at pH 7.8 our enzyme preparation had an activity of about 150 units/mg protein at pH 7.0.

Amino acid composition, molecular weight and subunit structure of aminoacylase

The amino acid composition of the enzyme is shown in Table II. From the amino acid composition a molecular weight of 85 500 is calculated. Analysis of the -SH content with Ellman reagent in the presence and absence of urea revealed four cysteine residues. Since 12 cysteic acids are found after performic acid oxidation (see below) 8 cysteine residues form 4 disulfide bridges. The enzyme contains 12 tryptophan residues. As we have shown previously [7] the chemical modification of only two tryptophan residues abolishes the catalytic activity of aminoacylase.

Molecular weight and subunit structure

Molecular weight determinations were performed by SDS polyacrylamide gel electrophoresis and gel-filtration. When the electrophoretic mobilities of the reference proteins (lysozyme, chymotrypsinogen, lactate dehydrogenase, catalase, β-galactosidase) and aminoacylase are plotted against the logarithms of the polypeptide chain molecular weights the relative mobility of the main band of aminoacylase polypeptide chain corresponds to a molecular weight of about 43 000. A weak band is found in the region of 86 000. The last value is in good agreement with the results of the Sephadex column chromatography (see below).

TABLE II

AMINO ACID COMPOSITION OF AMINOACYLASE

Values are average of four determinations

Amino acid	Number of residues per mol	Best integer	Mol percent
Ala	68.6 ± 0.6	68	8.79
Arg	39.4 ± 0.8	40	5.17
Asp	60.3 ± 0.4	60	7.75
Cys	12.1 ± 0.6	12	1.55
Glu	91.4 ± 1.2	92	11.89
Gly	59.4 ± 1.1	60	7.75
His	24.3 ± 0.4	24	3.10
Ile	22.4 ± 1.4	22	2.84
Leu	68.5 ± 0.9	68	8.79
Lys	34.6 ± 0.8	34	4.39
Met	16.6 ± 0.9	16	2.07
Phe	39.6 ± 0.7	40	5.17
Pro	51.7 ± 1.6	52	6.72
Ser	46.4 ± 1.4	46	5.94
Thr	43.3 ± 1.6	44	5.68
Trp	11.9 ± 0.7	12	1.55
Tyr	19.5 ± 0.6	20	2.58
Val	62.0 ± 1.0	62	8.01

From these experiments we must conclude that aminoacylase can be dissociated into two subunits. The dissociation occurs during SDS gel electrophoresis even without mercaptoethanol or performic acid treatment. The subunits are therefore not linked together by disulfide bonds. The subunit is enzymatically inactive; the active species with the lowest molecular weight is the dimer. In good agreement with SDS polyacrylamide electrophoresis were the results of a further molecular weight determination by gel filtration on Sephadex G 150. A value of about 86 000 is found for the active enzyme. Bruns and Schulze who determined the molecular weight for aminoacylase for the first time with the ultracentrifuge calculated a value of 76 500 from a sedimentation coefficient $S_{20} = 5.5 \cdot 10^{-3}$ s and a diffusion constant of $7.02 \cdot 10^{-7}$ cm$^2 \cdot$ s^{-1} [5].

Number, availability and reactivity of -SH groups and disulfide bonds

The state and function of the -SH groups and disulfide bonds of aminoacylase are of special interest since SH blocking reagents such as *N*-ethylmaleimide, carboxypyridine disulfide, vinylpyridine, and mercury compounds inactivate the enzyme as do disulfide reducing agents such as dithiothreitol and mercaptoethanol. The results of the experiments on the determination of the number of cysteine and cystine residues in the enzyme are summarized in Table III.

As can be seen from Table III aminoacylase contains 4 -SH groups which are freely available in the native enzyme for reaction with vinylpyridine but also with Ellman reagent, carboxypyridine disulfide or *N*-ethylmaleimide. After treatment with vinylpyridine about 4 residues of *S*-β[4-pyridylethyl]-cysteine per mol of enzyme are found by amino acid analysis. The number of -SH groups available with the above-mentioned reagents does not differ in the absence and presence of 8 M urea. However after treatment of the enzyme with

TABLE III

NUMBER OF CYSTEINE AND CYSTINE RESIDUES IN AMINOACYLASE

Values are the average of three experiments

Conditions	Number of cysteic acid residues after performic acid oxidation	Number of *S*-β[4-pyridylethyl]cysteine residues after treatment with vinylpyridine	Number of cysteine residues, best integer	Number of cystine residues, best integer	Number of ½-Cys residues, best integer
Native aminoacylase	12.1	—	—	—	12
Aminoacylase + vinylpyridine	8.3	4.2	4	4	12
Aminoacylase + 8 M urea + vinylpyridine	7.4	4.2	4	4	12
Aminoacylase + 8 M urea + dithiothreitol + vinylpyridine	4.3	7.5	8	2	12

dithiothreitol in the presence of 8 M urea the number of -SH groups rises to 8. That means that two disulfide bonds have been cleaved forming four additional -SH groups which could then be determined with the aid of vinylpyridine. In the absence of urea but in the presence of substrate, dithiothreitol also alters the number of -SH groups. After removal of the reducing agents by dialysis or gel filtration the -SH groups are rapidly reoxidized to disulfide bonds with recovery of activity. The total number of cysteine residues was determined after performic acid oxidation as cysteic acid with the amino acid analyzer. Since the enzyme contains 12 half-cystine residues of which only four can be directly transformed to *S*-β[4-pyridylethyl]-cysteine we suggest that aminoacylase has four disulfide bonds two of which can be cleaved by treatment with dithiothreitol in 8 M urea, and an additional two which are so deeply buried in the molecule that they are only accessible to performic acid oxidation. Cleavage of two disulfide bonds destroys the catalytic activity. These two bonds are however easily restored after removal of the reducing agent.

Since the enzyme molecule is built up from two subunits, which we assume to be identical, we believe that every subunit contains two freely available -SH groups and two disulfide bonds one of which is easily reduceable with inactivation of the enzyme; the second is only cleaved by performic acid oxidation. Disulfide bonds are not engaged in subunit interaction.

Following our determinations of the number of thiol groups and disulfide bonds we have studied the reactivity of the -SH groups and their importance for the catalytic process. As a measure of their reactivity we used the rate constants with different -SH-blocking agents. Thus we evaluated the second order rate constants for inactivation of aminoacylase by different -SH reactants. The results of these experiments are demonstrated in Table IV. The table shows

TABLE IV

SECOND ORDER RATE CONSTANTS OF THE INACTIVATION OF AMINOACYLASE BY DIFFERENT THIOL REAGENTS IN 0.1 M TRIS BUFFER pH 8.0 AT 25°C

Thiol reagent	$k_2^* \cdot 10^2$ ($l \cdot mol^{-1} \cdot s^{-1}$)
Iodoacetamide	6.26
1,3-dichloro-2-propanone	1.33
Chloroacetonitrile	0.55
2-hydroxy-5-nitrobenzylbromide	1.00
Ellman's reagent	360.00
Carboxypyridine disulfide	207.00
Vinylpyridine	3.67
N-ethylmaleimide	3950.00

* $k_2 = k_1/c$; k_1 = first order rate constant of inactivation; c = concentration of thiol reagent.

that *N*-ethylmaleimide, Ellman reagent and carboxypyridine disulfide are the most potent inhibitors of the enzyme. An unexpectedly low rate of inactivation was observed with the alkylating agents iodoacetamide and with Koshland's reagent (2-hydroxy-5-nitrobenzylbromide). The rate of inactivation of the enzyme does not necessarily reflect only the reactivity of the essential -SH groups but also provides no direct information on the stoichiometry of the inactivation process with respect to the number of -SH groups blocked. We therefore have measured directly the reaction rate of various -SH reagents with the different -SH groups of aminoacylase and studied the relation between the loss of -SH groups and the inactivation of the enzyme. Fig. 1 shows that modification by *N*-ethylmaleimide of one -SH group per subunit completely abolishes the catalytic activity of aminoacylase.

Of the two -SH groups present per subunit one is essential for the activity. The reactivity of this essential -SH group on each subunit is identical. We there-

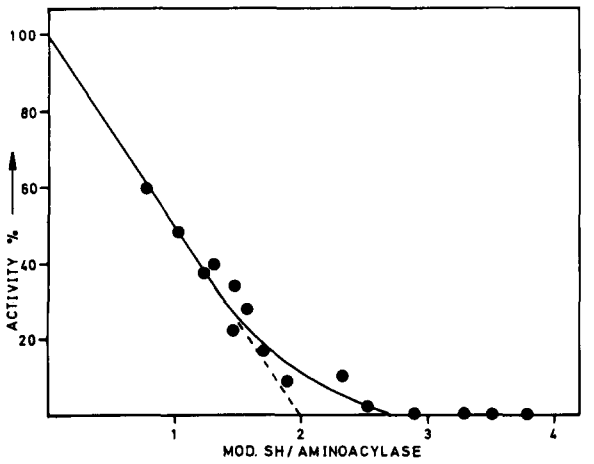

Fig. 1. Relation between activity and extent of modification of -SH groups of aminoacylase by *N*-ethylmaleimide.

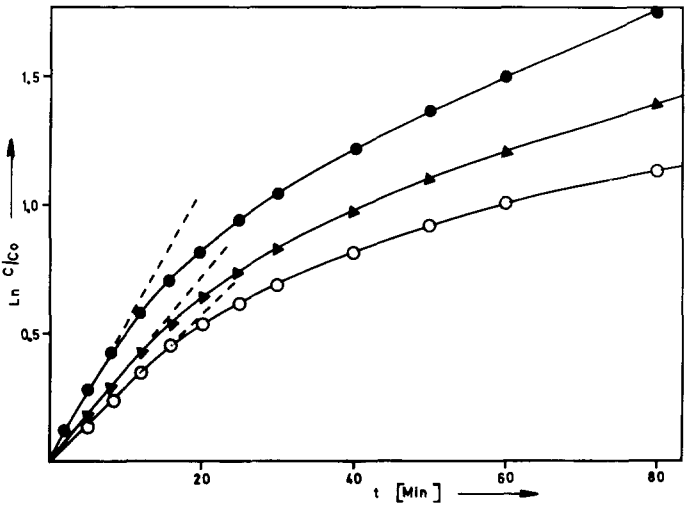

Fig. 2. Kinetics of the reaction of aminoacylase with Elmman reagent at pH 8.0, 25° C. ●———●, enzyme alone; ▲———▲, in the presence of 25 mM of the competitive inhibitor *N*-tosyl-L-alanine; ○———○, in the presence of 10 mM concentration of the competitive inhibitor D,L-norleucine.

fore conclude that the enzyme contains one active site per subunit. A differentiation between the essential and nonessential -SH groups is possible by measuring the rate of reaction with *N*-ethylmaleimide, Ellman reagent or carboxypyridine disulfide. As is shown in Fig. 2 a two phase reaction is observed.

The initial slope of the curves corresponds to the reaction rate of the fast-reacting essential -SH groups, the slope of the second part of the curve is a measure of the reaction rate of the two nonessential -SH groups. The effect of the competitive inhibitors *N*-tosyl-L-alanine and D,L-norleucine is also illustrated in Fig.2. The inhibitors reduce the reactivity of both classes of -SH groups. The second-order rate constants for the reaction of the variously reactive -SH groups with various reagents are summarized in Table V. The pH dependence of

TABLE V

SECOND-ORDER RATE CONSTANTS OF THE REACTION OF -SH GROUPS OF AMINOACYLASE WITH VARIOUS REAGENTS AT pH 8.0, 25° C

Thiol reagent	$k_2 \cdot 10^2$ ($l \cdot mol^{-1} \cdot s^{-1}$) (fast-reacting SH groups)	$k_2 \cdot 10^2$ ($l \cdot mol^{-1} \cdot s^{-1}$) (slow-reacting SH groups)
N-Ethylmaleimide	667 *	80 *
Carboxypyridine disulfide	207	61
Ellman's reagent	360	76
Ellman's reagent + 25 mM *N*-tosylalanine	225	55
Ellman's reagent + 10 mM D,L-norleucine	198	38

* at pH 7.0 and 0° C.

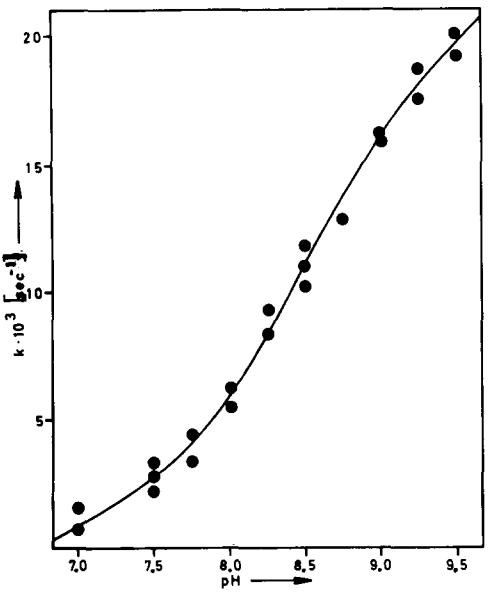

Fig. 3. pH dependence of the first-order rate constant of the reaction of aminoacylase with *N*-ethylmaleimide. A p*K* value of 8.35 is obtained from the curve.

the reaction of the essential -SH groups with *N*-ethylmaleimide was studied to determine the sulfhydryl p*K* values. Plotting the first-order rate constants against the pH gives Fig. 3. From this diagram a value of 8.35 is obtained for the p*K* of the essential -SH group of each subunit.

A comparison of the second-order constants for the reaction of *N*-ethylmaleimide with low molecular weight -SH compounds of different structure and the rate constants for reaction of the -SH groups of ficin, papain and aminoacylase is given in Table VI. These rate constants give information on the reactivity of the different -SH groups. From this table one recognizes that the reactivity of low-molecular-weight SH compounds with *N*-ethylmaleimide is much

TABLE VI

COMPARISON OF THE SECOND-ORDER RATE CONSTANTS OF THE REACTION OF SH COMPOUNDS AND SH ENZYMES WITH *N*-ETHYLMALEIMIDE AT pH 7.0

SH compound	$k_2 \cdot 10^{-3}$ ($l \cdot mol^{-1} \cdot sec^{-1}$)	Temp. (°C)	Relative reactivity	Ref.
Cysteine	1.53	25	115	16
N-acetylcysteine-amide	1.33	25	100	16
Glutathione	1.18	25	88	16
Mercaptomethyl-imidazole	9.45	25	710	16
Ficin	6.5×10^{-6}	25		17
Papain	1.85×10^{-3}	30		18
Aminoacylase	6.67×10^{-3}	0		

TABLE VII

EFFECT OF THIOL COMPOUNDS ON THE ACTIVITY OF AMINOACYLASE (% RESIDUAL ACTIVITY)

Concentration (M)	NaHS	Cysteine	Mercapto-ethanol	Dithiothreitol
$5 \cdot 10^{-5}$	98	90	60	44
$1 \cdot 10^{-4}$	94	80	46	25
$5 \cdot 10^{-4}$	90	60	28	5
$1 \cdot 10^{-3}$	75	30	16	0
$5 \cdot 10^{-3}$	25	12	0	0

higher than that of enzyme -SH groups and that the essential -SH groups of aminoacylase are more reactive than those of ficin and papain.

When studying the effect of thiol compounds on the activity of aminoacylase we observed in some cases a strong inhibition of the enzyme. This is illustrated in Table VII. Dithiothreitol is the strongest inhibitor and abolishes the activity completely at a concentration of $1 \cdot 10^{-3}$ M. The inhibitory action of the -SH compounds decreases with decreasing redox potential. As we have shown above the inactivation of the enzyme is a consequence of the cleavage of one disulfide bond per subunit. This disulfide bond is evidently essential for the maintainance of the active conformation. The stability of the enzyme decreases rapidly after cleavage of this disulfide bonds. These processes are the reason why it is not possible to protect reversibly the -SH groups of aminoacylase.

The reactivity of the thiol groups with various -SH reagents differs considerably as a consequence of the influence of the molecular environment. From our experiments on the substrate specificity and with competitive inhibitors [2] we know that aminoacylase has a strong hydrophobic binding center. It is therefore reasonable to assume that the essential -SH groups are located in or near this hydrophobic region. In accord with this assumption we find that competitive inhibitors such as D,L-norleucine and *N*-tosyl-L-alanine exhibit a marked protective effect on the reaction of the -SH groups. Somewhat surprising is the stability of the -SH groups against oxidation by oxygen. Purification and activity measurements are possible without protective -SH compounds in the buffers. On the contrary, such compounds inactivate the enzyme fairly rapidly by cleaving an "essential" disulfide bond. The p*K* values of the essential -SH groups are found in a normal range and do not differ considerably from the p*K* of the -SH group of cysteine, for instance.

Acknowledgements

The experiments were supported by a grant from Deutsche Forschungsgemeinschaft.

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