Identification of Essential Histidine Residues of Aminoacylase by Photooxidation and by Reaction with Diethylpyrocarbonate

Werner Kördel and Friedhelm Schneider

Physiologisch-Chemisches Institut II der Universität Marburg

State and function of the histidine residues of aminoacylase were investigated by photooxidation in the presence of methylene blue and by chemical modification with diethylpyrocarbonate. Complete inactivation of the enzyme was observed after oxidation of 4 histidine residues. From the pH dependence of the photooxidation it becomes evident that the inactivation of the enzyme is not a consequence of the simultaneous oxidation of tryptophan residues. The enzyme is also inactivated by chemical modification of histidine residues with diethylpyrocarbonate. Activity is restored by treatment with hydroxylamine. Zn^{2+}-ions which are essential for the activity of aminoacylase protect the available histidine molecules against photooxidation and attack by diethylpyrocarbonate. It is suggested that histidine is involved in the binding of the essential Zn^{2+}-ions.

Our previous studies on the active center and the catalytic mechanism of aminoacylase have shown that the enzyme has a strong hydrophobic binding center [1] and that at least two SH groups play an essential role in the catalytic process [2, 3]. We further found that chemical modification of two tryptophan residues [4] as well as substitution of the ε-amino groups of two lysine molecules abolishes the catalytic activity of the enzyme [3]. These results support our assumption that the enzyme, which is a dimer, contains two active sites [2].

In the present communication we report our results on the investigation of the state and function of the histidine residues of aminoacylase by photooxidation in the presence of methylene blue and by chemical modification with diethylpyrocarbonate. Since the function of the histidine residues of the enzyme are presumably closely related to the role of Zn^{2+}-ions of aminoacylase [3] we have also studied the effect of this metal ions on the activity of the enzyme and on the reactivity and accessibility of the histidine residues.

Materials and Methods

Aminoacylase

Pig kidney aminoacylase was a gift of Boehringer, Mannheim. The enzyme was purified by chromatography on Sephadex G 150 and DEAE cellulose up to a specific activity of 250 U/mg protein [2]. The purified enzyme was homogeneous as judged by gel- and immunoelectrophoresis.

Chemicals

Buffer substances, hydroxylammonium chloride, EDTA, o-phenanthroline, zinc acetate, hydrochloric acid supra pure, methylene blue were from Merck, Darmstadt. Ellman reagent 5,5'-dithiobis(2-nitrobenzoic acid) and diethylpyrocarbonate were from Serva, Heidelberg. N-chloroacetylalanine was synthesized according to Greenstein et al. [5].

Activity measurements

Activity measurements were performed spectrophotometrically with a Zeiss PMQ II or a Beckman Acta III spectrophotometer. Hydrolysis of N-chloroacetyl-L-alanin was followed at 238 nm in a 0.1 M phosphate-borate-buffer pH 8.0 at 40 °C [1, 4]. The reaction was started by addition of the native or modified enzyme $(10 - 20 \mu l)$ to a 12.5 mM solution of the substrate in a cuvette. ε_{238} of the substrate is $185 [1 \times mol^{-1} \times cm^{-1}]$.

Amino acid analysis

Protein hydrolysis for amino acid analysis was accomplished according to Matsubara and Sasaki [6] to prevent destruction of tryptophan. For amino acid analysis a Beckman Multichrom B Analyzer was used. For further details see [3].

Photooxidation experiments

Photooxidation experiments in the presence of methylene blue were performed as described in [7]. The enzyme was dialyzed against 0.1 M phosphate-borate-buffer pH 6 – 8. 1 ml samples containing 1 mg protein and 0.025% in methylene blue were irradiated at 30 °C. After appropriate irradiation

times the remaining activity was determined and the dye was removed by dialysis in the dark; the samples were then prepared for amino acid analysis as described in [2]. Free SH groups were determined with Ellman reagent [8].

Reaction of the enzyme with diethylpyrocarbonate (ethoxyformic anhydride) [9].

The enzyme was dialyzed against 0.1 M sodium acetate buffer pH 5.0 and diluted to a concentration of 0.5 mg/ml. Samples were incubated with 0.8 – 32 mM diethylpyrocarbonate for 30 min at room temperature. The remaining activity was determined and the difference spectra of the modified enzyme against the native protein in the range of 320 – 230 nm were recorded. For reactivation samples were dialyzed overnight at 4 °C against a 0.01 M Tris buffer pH 7.8 which was 0.1 M in hydroxylamine. Thereafter the activity and the absorption at 240 nm were measured spectrophotometrically.

Effect of zinc ions on the oxidation of histidine. To remove any metal ions the enzyme was dialyzed for 40 hours against a 0.1 M phosphate-borate-buffer pH 8.0 containing 1 mM o-phenanthroline or 10 mM EDTA. Controls were dialyzed under the same conditions in the absence of metal chelating agents. Aliquotes were photooxidized as described above and the activity was measured in the usual manner.

Effect of zinc ions on the reactivity of histidine molecules of aminoacylase with diethylpyrocarbonate.

Aminoacylase (2.5 mg/ml) was dialyzed overnight at 4 °C against a 0.1 M sodium acetate buffer which was 1 mM in o-phenanthroline. Controls were dialyzed against the same buffer without o-phenanthroline. The enzyme concentration was adjusted to 1 mg/ml with the same buffer and 0.5 ml samples were incubated with diethylpyrocarbonate concentrations from 0.1 – 25 mM for 12 hours at 4 °C. The activity of the modified enzyme was determined. Zinc free samples were incubated with 1 mM Zn^{2+} before activity measurements.

Results and Discussion

Dye sensitized photooxidation of aminoacylase

Aminoacylase is irreversibly inactivated by illumination in the presence of methylene blue and oxygen. To obtain some general information on the sensitivity of those aminoacids which potentially are photooxidized in the presence of methylene blue [10] (histidine, tyrosine, tryptophan, methionine and cysteine) the relation between the extent of oxidation of these aminoacids in the enzyme and the time

of irradiation was studied at pH 7.5. The results of these experiments are shown in Fig. 1, from which the following conclusions may be drawn: No tyrosine molecule from 20 present in the enzyme [2, 3] is available for oxidation under the conditions chosen; methionine residues are scarcely affected and surprisingly also the SH-groups of the protein are only slowly oxidized. The most reactive amino acids are
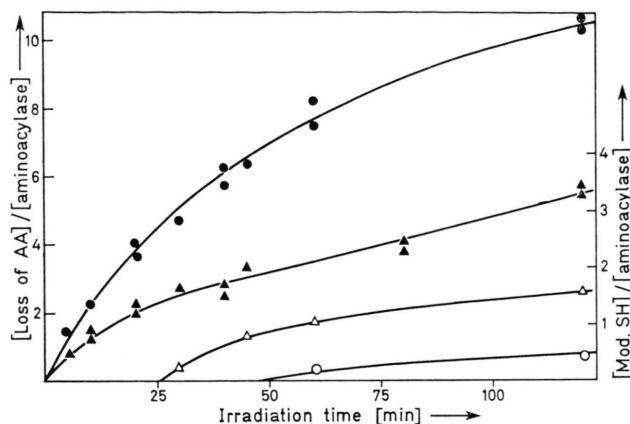

Fig. 1. Relation between photooxidation of histidine ● – ●, tryptophan ▲ – ▲, cysteine △ – △, and methionine ○ – ○ of aminoacylase and time of irradiation. Experiments were carried out as described under methods.

histidine and tryptophan; during the whole period of reaction about twice as much histidine as tryptophan molecules are destroyed. Since aminoacylase contains 12 tryptophan and 24 histidine residues [2, 3], it follows that both amino acids are approximately equally reactive. In previous experiments [4] we have shown that chemical modification of two tryptophan residues by diazo-1-H-tetrazole or N-bromosuccinimide abolishes the catalytic activity of the enzyme. We therefore have analyzed whether the oxidation of histidine or tryptophan is responsible for the inactivation of the enzyme. This question may be answered from experiments on the pH dependence of the photooxidation of aminoacylase. The results of these experiments are shown in Fig. 2. A close relation between the loss of histidine and the inactivation of the enzyme is observed; the rate of photooxidation of histidine and of inactivation of the enzyme increases with increasing pH. A "normal" pK of 7.0 ± 0.2 results from the pH dependence of the rate of the oxidation of histidine (Fig. 3). The correlation between inactivation of aminoacylase and oxidation of histidine
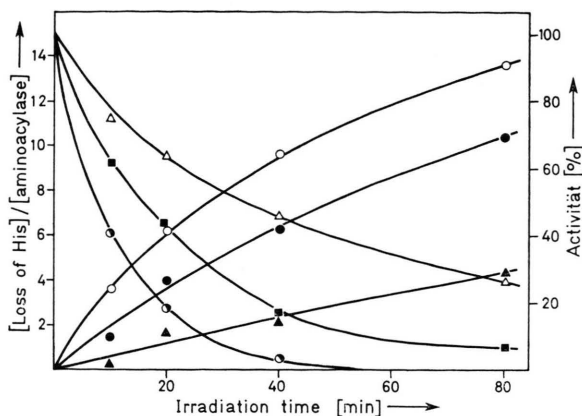
Fig. 2. Relation between histidine oxidation and inactivation of aminoacylase respectively and time of irradiation at different pH. Loss of histidine at pH 6.0 ▲ — ▲, pH 7.0 ● — ●, pH 8.0 ○ — ○. Remaining activity at pH 6.0 △ — △, pH 7.0 ■ — ■, pH 8.0 ◑ — ◑. Conditions see Fig .1.
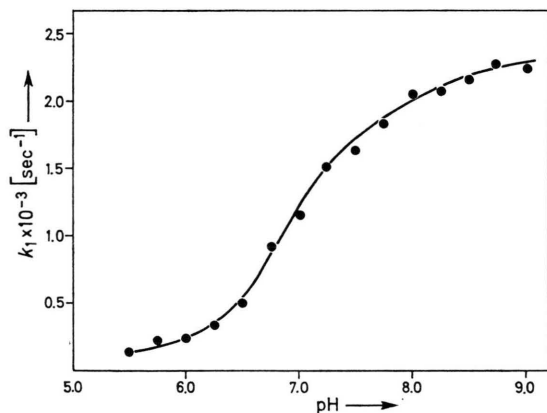

Fig. 3. Effect of pH on the rate of photooxidation of histidine. Apparent first order rate constants were determined from plots lg his_0/his_t versus time.
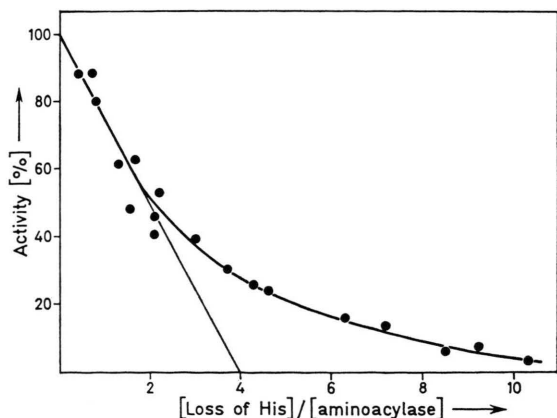

Fig. 4. Relation between remaining activity of aminoacylase and loss of histidine by photooxidation at pH 7.5.

residues (Fig. 4) shows that complete inactivation takes place after loss of 4 histidine molecules. Since oxidation of essential SH groups does not occur to a measureable extend before destruction of 5 histidine residues, the importance of tryptophan modification for the loss of activity must be clarified. From Fig. 5 it becomes evident that between pH 6 and 8 the oxidation of tryptophan is pH independent. The pH dependence of the inactivation of the enzyme by photooxidation can therefor not be a consequence of the destruction of tryptophan; it is the result of the loss of histidine residues. From the results of these experiments we must further conclude that the tryptophan residues which are modified by diazo-1-H-tetrazole [4] and by photooxidation are not identical because a remaining activity of about 50% is found after oxidation of 2 tryptophan and 2 histidine residues at pH 6. The complete inactivation of aminoacylase by photooxidation is therefore a consequence of the oxidation of 4 histidine residues.
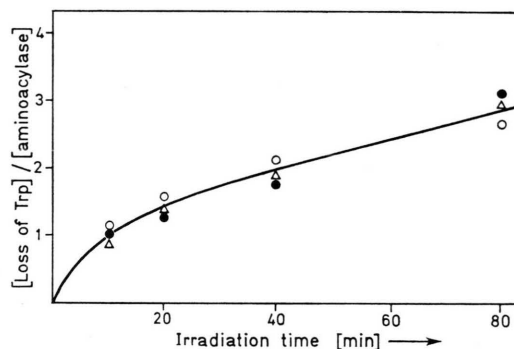

Fig. 5. Relation between tryptophan oxidation and time of irradiation at differing pH values. pH 6.0 ● — ●, pH 7.0 △ — △, pH 8.0 ○ — ○. Conditions see above.

Reaction of aminoacylase with diethylpyrocarbonate

To confirm the results of the photooxidation experiments with respect to the effect of histidine modification we have further studied the reaction of aminoacylase with diethylpyrocarbonate. The reaction of this reagent with imidazole groups of proteins is accompanied by an increase in absorbance at $230-240$ nm [9]. The relation between the increase of the absorbance at 240 nm and the loss of activity is illustrated in Fig. 6. A nearly linear decrease of activity is observed with increasing absorbance at 240 nm. Because of some

intertainties in the molar extinction coefficient of the ethoxyformyl imidazole [11] we have not quantitated the reaction. Reactivation of the enzyme can easily be accomplished by deacylation of the modified histidine residues with hydroxylamine at neutral pH. The results of these experiments are also demonstrated in Fig. 6. Since the reaction is selective with accessible imidazole groups under the conditions chosen (modification of tyrosine residues could not be detected) the results of these studies confirm the essential role of histidine residues for the catalytic activity of the enzyme.
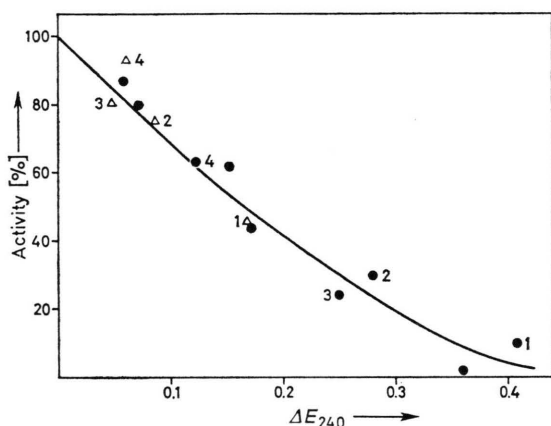

Fig. 6. Decrease of activity of aminoacylase with increasing absorption at 240 nm as parameter for the formation of ethoxyformyl histidine ●—●. Reactivation by treatment with hydroxylamine △—△.

Effect of zinc ions on the photooxidation of histidine residues of aminoacylase

The activity of aminoacylase depends on the presence of zinc ions [3]. Since it is well known that imidazole groups of histidine are often involved in the binding of metal ions in proteins [12] we have compared the rate of photooxidation of histidine and the inactivation of the enzyme in the presence and absence of zinc ions. Photooxidation experiments were therefore performed with enzyme preparations extensively dialyzed against metal chelating agents such as o-phenanthroline or EDTA. These enzyme preparations, which are enzymatically inactive can easily be reactivated by addition of zinc ions [3]. The rate of photooxidation of histidine molecules of aminoacylase and of the inactivation of the enzyme in the presence and absence of zinc ions are demonstrated in Fig. 7. The

remaining activity of the metal free photooxidized samples was determined after addition of zinc ions. From Fig. 7 one recognizes that at the beginning of the irradiation the loss of histidine is by far faster in the absence of metal than in the zinc containing samples. After some minutes the rate of oxidation of the imidazole groups runs nearly parallel in both experiments. Approximately the same course is observed with respect to the inactivation of the enzyme in the presence and absence of zinc. The relation between the rate of oxidation of histidine residues and the remaining activity of aminoacylase with and without zinc ions is shown in Fig. 8. In both cases extrapolation of the initial linear decrease of activity intersects the abscisse at 4 histidine residues.
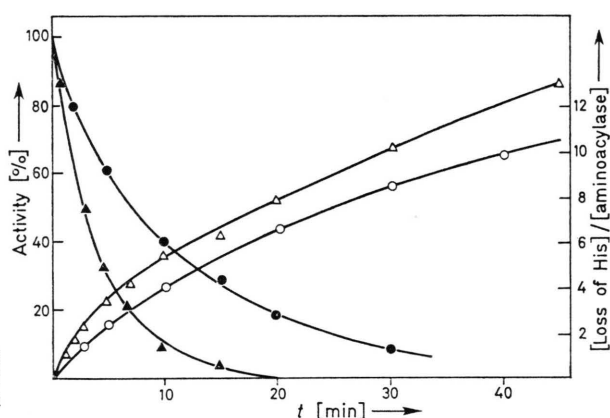

Fig. 7. Loss of activity of aminoacylase by photooxidation in the presence ●—● and absence ▲—▲ of zinc ions. Number of oxidized histidine residues in the presence ○—○ and absence △—△ of zinc ions.
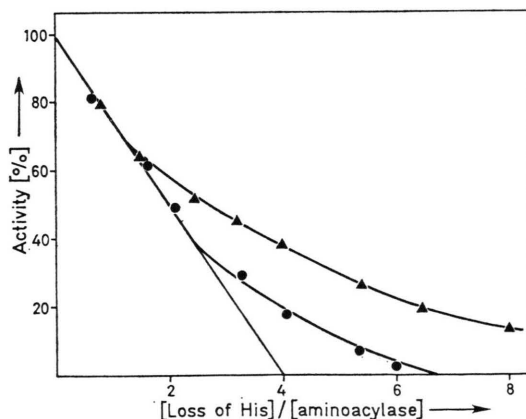

Fig. 8. Relation between the loss of activity of aminoacylase and the number of modified histidine residues in the presence ▲—▲ and absence ●—● of zinc ions.

While the accessibility of the histidine residues of aminoacylase is obviously not influenced by the metal, the rate of oxidation of the essential residues is significantly reduced by zinc ions.

Fig. 8 shows further, that the deviation from the straight line begins significantly later with the zinc free enzyme, implying the essential histidine residues being preferentially oxidized during the first period of irridation in the absence of zinc ions. From these results we may conclude that zinc ions protect essential histidine residues against dye sensitized photooxidation. At this stage of investigation we cannot decide however whether this protective effect is caused by a general stabilisation of the conformation of the enzyme by zinc ions or by a specific interaction between imidazole groups and zinc ions.

Effect of zinc ions on the rate of inactivation of aminoacylase by diethylpyrocarbonate

Zinc ions do not only protect the histidine residues of the enzyme against photooxidation but they also protect the enzyme against inactivation by diethylpyrocarbonate. This was demonstrated in inactivation experiments of aminoacylase by diethylpyrocarbonate in the presence and absence of zinc ions. The results of these experiments are shown in Fig. 9 illustrating the loss of activity after incubation of the enzyme with increasing concentrations of diethylpyrocarbonate in the presence and absence of zinc ions. While the inactivation of the zinc containing enzyme is measurable only above 1 mM
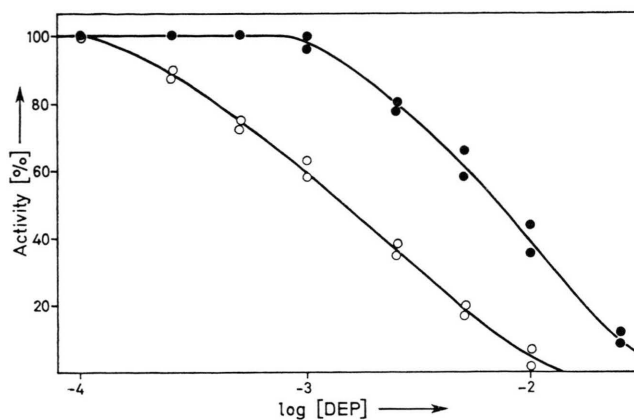

Fig. 9. Effect of zinc ions on the inactivation of aminoacylase by diethylpyrocarbonate. ●—● in the presence of zinc ions; ○—○ in the absence of zinc ions.

diethylpyrocarbonate zinc free aminoacylase has already lost about 40% of its activity at the same concentration of the modification reagent. Since the loss of activity of the enzyme is a consequence of the modification of histidine residues, the protective effect of zinc ions against the attack of diethylpyrocarbonate on essential histidine molecules is evident.

A quantitative study on the relation between zinc concentration and activity of aminoacylase and the effect of zinc ions on the reactivity of the essential SH groups of the enzyme is described in a next paper.

[1] W. Kördel and Fr. Schneider, Hoppe-Seyler's Z. Physiol. Chem. **356**, 915—920 [1975].
[2] W. Kördel and Fr. Schneider, Biochim. Biophys. Acta **445**, 446—457 [1976].
[3] W. Kördel, Thesis University of Marburg, 1976.
[4] W. Kördel and Fr. Schneider, Hoppe-Seyler's Z. Physiol. Chem. **375**, 1109—1115 [1976].
[5] P. J. Fodor, V. E. Price, and J. P. Greenstein, J. Biol. Chem. **178**, 503—509 [1949].
[6] H. Matsubara and R. M. Sasaki, Biochem. Biophys. Res. Commun. **35**, 175—178 [1969].

[7] W. J. Ray Jr., Methods in Enzymology, **Vol. XI,** pp. 490 —498 (C. H. Hirs, ed.), Academic Press, New York 1967.
[8] G. L. Ellman, Arch. Biochem. Biophys. **82**, 70—77 [1959].
[9] G. E. Means and R. E. Feeney, Chemical Modification of Proteins, pp. 81—83, Holden Day, In., London.
[10] L. Weil, W. G. Gordon, and A. R. Buchert, Arch. Biochem. Biophys. **34**, 1—8 [1951].
[11] Chr. J. Dickenson and F. M. Dickinson, Europ. J. Biochem. **52**, 595—603 [1974].
[12] R. J. Sundberg and R. B. Martin, Chem. Reviews **74**, 471—515 [1974].