PHOTOSENSITIZED OXIDATION OF ASPARAGINE-GLUTAMINE DEAMIDASE FROM *Pseudomonas fluorescens*

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After the discovery of the antitumor activity of asparaginase EC-2 from *Escherichia coli* a number of microbial deamidases with a similar action have been obtained. An asparaginase producer, identified as *Pseudomonas fluorescens* [2], was isolated previously in the writers' laboratory and a deamidase which catalyzes the hydrolysis of asparagine and glutamine, and which possesses antitumor activity, was isolated from it in a homogeneous form [3, 4]. The substrate specificity of the enzyme [1] and its quaternary structure have been established and its molecule has been shown not to contain free sulfhydryl amino-acid residues essential for the catalytic activity of the enzyme. One method whereby the participation of certain amino acids in the act of catalysis can be demonstrated is photosensitized oxidation. In this case residues of tryptophan, histidine, tyrosine, methionine, and cysteine are oxidized most readily.

To examine the role of these amino-acid residues it was decided to study photooxidation of homogeneous asparagine-glutamine (AG) deamidase from *Pseudomonas fluorescens*.

EXPERIMENTAL METHOD

Culture of the microorganism and purification of AG deamidase were carried out as described previously [3]. During photooxidation a solution of the enzyme in 0.05 M K-phosphate buffer, pH 8.0, containing the sensitizer, was exposed to the light of a 100-W incandescent lamp at a distance of 15 cm and at room temperature. Aliquots were taken at definite time intervals from the samples and immediately freed from sensitizer by gel-filtration on a column with Sephadex G-25, equilibrated with 0.05 M K-phosphate buffer, pH 8.0. Enzyme activity was determined by using L-asparagine or L-glutamine as substrates [3]. The protein concentration in the solution was determined by Lowry's method [7]. Fluorescence of the protein was measured on a Hitachi MPF-2A spectrofluorometer (Japan). The amino-acid composition of the protein was determined after hydrolysis with 4 N β-toluenesulfonic acid on an LKB-3201 automatic analyzer (Sweden). The tryptophan content in the protein was measured spectophotometrically [6].

EXPERIMENTAL RESULTS

Methylene blue (0.005%) or Bengal Rose (0.01%) were used as sensitizers. During illumination of a 10^{-6} M solution of the homogeneous deamidase in 0.05 M K-phosphate buffer, pH 8.0, virtually complete inactivation of the enzyme took place. When methylene blue was used, the time taken was about 30 min, compared with 70 min with Bengal Rose. In the absence of sensitizers illumination of the enzyme solution did not cause any change in its activity, as was also the case when it was incubated with sensitization in darkness. The fall in glutaminase and asparaginase activity took place parallel with each other, thus confirming the existence of a single catalytic center of the deamidase.

The fall in activity was not connected with a disturbance of the quaternary structure of the enzyme, for the completely inactivated enzyme had the same electrophoretic mobility in polyacrylamide gel as the native deamidase.

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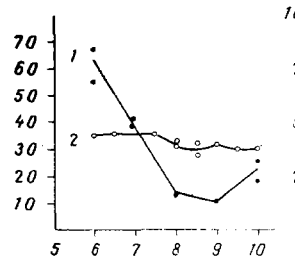

Fig. 1

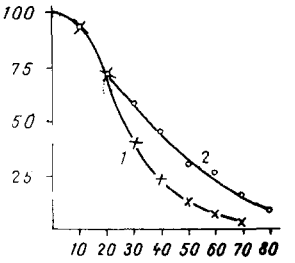

Fig. 2

Fig. 1. Dependence of degree of inactivation of AG deamidase on pH during photooxidation. Duration of illumination 20 min. Oxidation with: 1) methylene blue, 2) Bengal Rose. Abscissa, pH; ordinate, inactivation of enzyme (in %).

Fig. 2. Effect of duration of photooxidation on AG deamidase activity and its fluorescence. 1) Enzyme activity, 2) intensity of fluorescence. Abscissa, time (in min); ordinate, residual activity and fluorescence of protein (in %).

TABLE 1. Effect of Photooxidation with Methylene Blue on Amino Acid Composition of AG Deamidase

Amino acid residues	Number of residues in molecule, residual activity, %		
	100	50	10
Tryptophan *	8	7	6
Histidine	14	13	7
Methionine	29	30	27
Tyrosine	27	27	29

Legend. Asterisk indicates that results were obtained spectrophotometrically.

Dependence of the velocity of photoinactivation on pH is shown in Fig. 1. When Bengal Rose was used as sensitizer it was practically independent of pH within the range 6.0-10.0. However, when methylene blue was used, there was a marked increase in the velocity of enzyme inactivation in the pH region 8.0-9.0, within which the enzyme exhibits maximal activity. Comparison of these results with those of the experiments of Westhead [9], who studied the effect of pH on the velocity of oxidation of free amino acids in the presence of Bengal Rose, suggests that tryptophan residues play a definite role in the catalytic activity of the deamidase. Accordingly, the next step was to study the effect of photooxidation of the enzyme with Bengal Rose on fluorescence of the protein (Fig. 2). When fluorescence was excited by monochromatic light with wavelength 290 nm, the maximum of the emission spectrum was at 345 nm, which is characteristic of tryptophan residues in a hydrophilic environment. In the course of photooxidation the intensity of fluorescence diminished but the character of the emission spectrum was unchanged. During the first 10 min of photooxidation the decrease in catalytic activity of the enzyme and in the intensity of fluorescence of the tryptophan residues followed a parallel course, but later the velocity of inactivation became greater than that of oxidation of tryptophan residues. This suggests that inactivation was connected with oxidation not only of tryptophan residues, but also of other amino acid residues. To identify them, the deamidase when inactivated to different degrees in the course of photooxidation, was subjected to amino acid analysis, and the results are given in Table 1. In the course of the first 5 min of photooxidation activity of the enzyme decreased by 50%, and this was accompanied by oxidation of 12.5% of tryptophan and 7% of histidine residues. With a fall in enzyme activity of 10% of the initial value the number of oxidized tryptophan residues was 25% and the number of oxidixed histidine residues was 50%. No decrease was found in the number of tyrosine and methionine residues contained in the protein, or of any other amino acids. The resistance of the tyrosine and methionine residues to oxidation is most probably attributable to the fact that they lie in hydrophobic regions of the molecule.

These results suggest that during photooxidation the first event to take place is destruction of tryptophan residues, which are evidently on the surface of the protein globule. This leads to partial disturbance of the conformation of the enzyme molecule, accompanied by a fall in its activity, and it makes the histidine residues accessible for oxidation. The essential role of histidine residues in the manifestation of enzymic activity of the asparaginase of *E. coli* has been demonstrated by photooxidation, chemical modification [7], and by the use of a kinetic approach [8].

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CLASSIFICATION OF XENOBIOTICS BY LOCALIZATION OF THEIR ACTION ON MITOCHONDRIAL ENZYME SYSTEMS

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KEY WORDS: xenobiotics; mitochondria; respiratory chain; mechanisms of action.

The study of the action of toxic agents on energy metabolism in biological objects is finding ever-increasing application in different branches of toxicology and pharmacology. These investigations have proved productive not only from the theoretical (to study the structure and functions of the respiratory chain and the mechanism of action of xenobiotics), but also from the practical point of view (for the rapid assessment of the toxicity of new compounds, establishing health rules, and so on) [1, 6, 9, 11-14, 16, 17, 19].

Analysis of existing data on the action of some hundreds of xenobiotics, of varied structure, on respiration and oxidative phosphorylation has led to the discovery of correlation between the chemical structure of the substance and the character and location of its action on molecular targets of the mitochondria. The results of one such analysis of personal data (over 100 compounds) and of data in the literature are given below.

EXPERIMENTAL METHOD

Experiments were carried out on liver mitochondria from male noninbred albino rats. Mitochondria were isolated and respiration recorded by a polarographic method as described in [8]. The test compounds were introduced into the spectrophotometer cell containing mitochondria in a volume of 1-10 µl. When nonaqueous solvents (ethanol, dimethyl sulfoxide, acetone) were used a control was set up with the pure solvents. Succinic and β-hydroxybutyric acids in the form of neutralized solutions were used as oxidation substrates.

EXPERIMENTAL RESULTS

The different stages of the complex and multistage process of oxidative phosphorylation,

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