PHOTOINACTIVATION OF NAD-GLUCOHYDROLASE FROM RABBIT HEART

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Irradiation of preparations of NAD-glucohydrolase from the rabbit heart with visible light in the presence of Bengal rose as sensitizer is accompanied by inactivation of the enzyme. The degree of inactivation depends on the length of exposure and the concentration of the dye. The substrates (NAD and NADP) protect the enzyme considerably against photoinactivation. The character of the relationship between the rate of photoinactivation of the enzyme preparations and the pH of the medium and also the absence of inactivation of the enzyme when pyridoxal-5'-phosphate is present as a sensitizer suggest that loss of activity of NAD-glucohydrolase is not connected with changes in the histidine residues in the protein molecule. SH groups likewise do not play an essential role in the exhibition of activity of this enzyme.

An important role in the conversion of the nicotinamide coenzymes is played by NAD-glucohydrolase (3.2.2.5), an enzyme catalyzing the hydrolysis of nucleotides with liberation of nicotinamide. In recent years the enzyme has been isolated in a highly purified form from many animal tissues [2, 9, 10], although the kinetic properties of NAD-glucohydrolases isolated from different sources, their amino-acid composition and their molecular weight have been found to be very different. The role of individual amino-acid residues in the mechanism of the catalytic action of the NAD-glucohydrolases is still an unsolved problem.

To continue the study of the properties of NAD-glucohydrolase from heart muscle in order to identify the functional groups of the enzyme, the effect of photooxidation in the presence of Bengal rose and pyridoxal-5'-phosphate on the enzymic hydrolysis of NAD and NADP was studied.

EXPERIMENTAL METHOD

Experiments were carried out on preparations of NAD-glucohydrolase isolated from rabbit heart muscle as described previously. Samples for incubation contained 0.07 M maleate buffer solution, pH 6.2, and various amounts of nicotinamide coenzymes and protein (indicated in the captions to the appropriate figures). The samples were incubated for 30 min at 37°C. The reaction was stopped by heating for 2 min in a boiling water bath. The loss of nicotinamide nucleotides by their enzymic reduction with alcohol dehydrogenase and glucose-6-phosphate dehydrogenase was determined in protein-free filtrates. In the experiments to study the effect of thiol reagents on NAD-glucohydrolase activity the loss of NAD was determined by the cyanide method [5]. Photooxidation of the enzyme was carried out in cells lying 15 cm from the source of light (a 200-W incandescent lamp) at 0°C. The volume of the sample irradiated was 0.7 ml. Bengal rose or pyridoxal-5'-phosphate was used as photosensitizer. At the end of illumination a solution of the substrate (NAD or NADP) was added to the samples and, after incubation at 37°C, the activity of the enzyme was determined. In the experiments to study protection by the substrates, the samples were illuminated in the presence of NAD and NADP.

As controls for the experimental samples, similar samples, one of which contained the same quantity of photosensitizer but was not illuminated, while the other was illuminated in the absence of the dye, were

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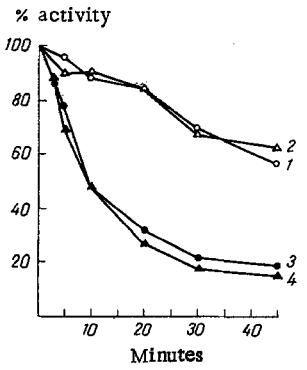

Fig. 1

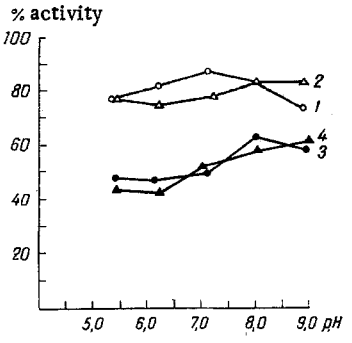

Fig. 2

Fig. 1. Photoinactivation of NAD-glucohydrolase in the presence of Bengal rose. Composition of sample illuminated: maleate buffer 0.07 M, protein 0.46 mg/ml, Bengal rose 3×10^{-6} M, pH 6.2. Volume of sample 0.7 ml. Abscissa, time of illumination; ordinate, activity (in %). Protein was illuminated in the presence of 0.69 mM NAD or 0.40 mM NADP as substrates; illumination (1, 2) in absence of substrates (3, 4), of NAD (1,3), and NADP (2, 4).

Fig. 2. Rate of photooxidation of NAD-glucohydrolase in the presence of Bengal rose at different pH values. Abscissa, pH; ordinate, activity (in %). Photooxidation of enzyme was carried out in 0.08 M tris-maleate buffer, protein concentration 0.65 mg/ml, Bengal rose 4×10^{-6} M (curve 1 – NAD, curve 2 – NADP) and 8×10^{-6} M (curve 3 – NAD, curve 4 – NADP).

used. The dye was added in a dark room, lit only by a flashlight with red or green filter for use with Bengal rose or pyridoxal-5'-phosphate, respectively. In the experiments to study the relationship between the rate of photoinactivation of the enzyme and pH, after the end of illumination aliquot portions were taken from the mixture and added to the samples for incubation containing 0.1 M maleate buffer, pH 6.0, and the corresponding substrates. In the experiments in which pyridoxal-5'-phosphate was used as the photosensitizer the composition of the samples was as follows: 0.05 M tris-HCl buffer, pH 8.0; protein 340 μg, and 1 mM pyridoxal-5'-phosphate. After illumination of the samples they were treated with NAD(NADP) solution made up in 0.12 M maleate to adjust the pH of the experimental samples to 6.2-6.3, at which incubation was carried out.

In the experiments to study the effect of thiol reagents the enzyme was preincubated at 37°C with PCMB for 1 h (tris-HCl buffer, pH 7.2), with n-ethylmaleimide (EMI) for 3 h (maleate buffer, pH 6.9), and with 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) for 1 h (tris-HCl buffer, pH 8.0).

EXPERIMENTAL RESULTS AND DISCUSSION

During irradiation of NAD-glucohydrolase isolated from heart muscle by visible light in the presence of Bengal rose as photosensitizer considerable loss of enzymic activity was observed as regards both hydrolysis of NAD and of NADP (Fig. 1). The degree of inactivation, as Fig. 1 shows, depended on the duration of illumination. Irradiation of the preparation for 45 min in the presence of 3×10^{-6} M Bengal rose reduced the activity of the enzyme by 85%. The sharpest changes in activity with respect to hydrolysis of both substrates took place during the first 10 min of illumination. In the subsequent time intervals the enzyme was inactivated more slowly. In the presence of substrates the process of photoinactivation of the enzyme was much less marked (Fig. 1). Presumably the substrates screened the photoreactive residues of the amino acids of the active center and thus protected them. The comparatively slight differences in the depth of inactivation and also in the shape of the curves obtained during hydrolysis of NAD and NADP point to the similarity between, or possible identity of, the chemical structure of the contact sites of NAD-glucohydrolase responsible for interaction with NAD and NADP.

TABLE 1. Effect of Thiol Reagents on NAD-Glucohydrolase Activity

Reagent	Concentration	% activity	
		NAD	NADP
PCMB	$1.4 \cdot 10^{-3}$	85	95
	$1 \cdot 10^{-3}$	89	97
EMI	$1 \cdot 10^{-2}$	90	87
	$5 \cdot 10^{-3}$	93	98
	$3 \cdot 10^{-3}$	94	99
DTNB	$1 \cdot 10^{-3}$	100	100

Since the reaction of photooxidation is insufficiently specific and several amino acids are oxidized, in order to identify the amino-acid residues responsible for the loss of enzymic activity experiments were carried out to study the rate of photoinactivation of the enzyme as a function of the pH of the medium. The rate of photooxidation of histidine, whether free or as a component of the enzyme, is known to depend on the degree of its protonation, and it rises sharply with an increase in pH [1]. As Fig. 2 shows the degree of inactivation of the enzyme in the presence of Bengal rose was substantially unchanged during an increase in pH from 5.0 to 9.0. However, there are indications in the literature that the absence of any clearly defined relationship between the rate of photooxidation and pH cannot be regarded in every case as a reliable criterion of the role of histidine as functional group in the active center of the enzyme [4]. Accordingly a special series of experiments was carried out in which pyridoxal-5'-phosphate, which is highly specific toward histidine [3, 6], was used as the photosensitizer. As these experiments showed, illumination of NAD-glucohydrolase from heart muscle in the presence of 1 mM pyridoxal-5'-phosphate for 40 min did not reduce the hydrolysis of either NAD or NADP. These results, like those of experiments to study the dependence of the degree of photoinactivation of the enzyme on pH (Fig. 2), suggest that inactivation of NAD-glucohydrolase in the presence of Bengal rose is not connected with destruction of the histidine residue in the molecule of the photooxidized protein. To test the possible participation of other photoreactive amino-acid residues, such as cysteine, in the catalytic process the enzyme was treated with reagents for SH groups. The results show that PCMB, EMI, and DTNB, even if taken in high concentrations, had only a weak inhibitory effect (Table 1). The very slight inhibition of NAD-glucohydrolase by high concentrations of PCMB and EMI observed in these experiments are more likely to be explained as a manifestation of the nonspecific action of the thiol reagents than by their blocking of the SH groups of the protein molecule [7, 8]. SH groups probably do not play an essential role in the exhibition of the catalytic activity of the enzyme. In this respect NAD-glucohydrolase from heart muscle resembles several of the glucohydrolases isolated from other animal tissues.

It can be postulated that an essential role in the exhibition of the catalytic activity of this enzyme is more likely to be played by residues of tryptophan, tyrosine, or methionine, which are also destroyed by photooxidation. However, before this problem can be finally solved direct evidence based on determination of the amino-acid composition of NAD-glucohydrolase before and after photoinactivation is required.

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