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CHEMICAL MODIFICATIONS OF THE CRYSTALLINE QUINOLINATE PHOSPHORIBOSYLTRANSFERASE FROM HOG LIVER *

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Summary

Amino acid analysis and chemical modification of the crystalline quinolinate phosphoribosyltransferase (EC 2.4.2.19) from hog liver were performed. The enzyme contained 29 residues of half cystine per mol. The enzyme activity was strongly inhibited by sulfhydryl reagents. The number of reactive (exposed) sulfhydryl group was determined to be 10.2 and total sulfhydryl group was to be 25.2 per mol by using 5,5'-dithiobis(2-nitrobenzoic acid). The enzyme activity was also inhibited by lysine residue-, histidine residue-, and arginine residue-modifying reagents. These results and the effect of preincubation with the substrates on chemical modifications suggest that the lysine residue, histidine residue and sulfhydryl group may be closely related to the binding site of quinolinic acid.

Introduction

The de novo biosynthesis of NAD proceeds from either tryptophan or aspartate and glycerol (or related compound to glycerol). The tryptophan-NAD pathway is found in animals [1,2] and the aspartate-NAD pathway is in plants [3] and microorganisms [4—6]. The former pathway has been verified but the latter has not been clearly determined. In both cases, quinolinic acid (pyridine-2,3-dicarboxylic acid) is known to be a common intermediate.

Quinolinate phosphoribosyltransferase (EC 2.4.2.19) catalyzes the stoichiometric conversion of quinolinic acid to nicotinic acid mononucleotide and carbon dioxide in the presence of 5-phosphoribosyl-1-pyrophosphate (*P*-Rib-*P*-*P*) and magnesium ion. This enzyme holds an important position in the de

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novo NAD biosynthetic pathways. The enzyme has been purified from beef liver [7,8], a pseudomonad [9] and caster bean endosperm [10], and their general properties have been reported, respectively. No information, however, on the active center of the enzyme protein has been available as far as we know. The previous paper [11] from this laboratory has shown that the crystalline enzyme preparation was isolated from hog liver at a high yield and was homogeneous in ultracentrifugal analysis and disc electrophoresis. The molecular weight of this enzyme was estimated to be 172 000 and that of the subunit to be 34 200 [11].

The present paper deals with the amino acid composition of the crystalline quinolinate phosphoribosyltransferase from hog liver, and the effects of various group-specific reagents on the enzyme in order to investigate the amino acid residues around the active center of the enzyme protein.

Materials and Methods

Enzyme

Quinolinate phosphoribosyltransferase was purified and twice crystallized from hog liver by methods described in a previous paper [11].

Chemicals

[2,3,7,8-^{14}C]Quinolinic acid was synthesized from [U-^{14}C]aniline and glycerol [12] at the Daiichi Pure Chemicals Co., Ltd., Tokyo. *P*-Rib-*P*-*P* (tetrasodium salt; purity: 90—95%), diisopropyl fluorophosphate and 2-hydroxy-5-nitrobenzyl bromide were purchased from Sigma Chemical Company. *p*-Chloromercuribenzoate, *N*-ethylmaleimide, 5,5′-dithiobis(2-nitrobenzoic acid), monoiodoacetic acid, β-naphthoquinone-4-sulfonic acid, 2,4,6-trinitrobenzene sulfonic acid, diethylpyrocarbonate, methylene blue, *p*-diazobenzenesulfonic acid, glyoxal and acetylimidazole were obtained from Nakarai Chemicals, Ltd., Kyoto.

Amino acid analyses

The crystalline enzyme preparation was dialyzed for 44 h against deionized distilled water (40 000-fold volume) at 4°C. The deionized enzyme solution was lyophilized, then the dried matter was thoroughly desiccated over phosphorus pentoxide powder in a vacuum desiccator. After being kept overnight at room temperature, the temperature of the desiccator was raised to and maintained at 105°C for 1 h. The completely dehydrated enzyme protein was weighed on a microbalance, then was used for successive analyses. A given amount of the enzyme protein (0.5122 mg) was dissolved in 1 ml of 6 M hydrochloric acid. The test tube containing this solution was sealed under vacuum. Hydrolysis was carried out at 110°C for 22 h. After hydrolysis, the hydrochloric acid was removed by using a rotary evaporator at 40°C for 30 min. Then the whole was placed in a vacuum desiccator containing sodium hydroxide pellets for 2 h. The hydrolysate was dissolved in 2 ml of water after which it was injected into a Hitachi amino acid analyzer, model KLA-5. Half cystine and methionine contents were determined as cysteic acid and methionine sulfone, respectively, by performic acid oxidations. The tryptophan con-

tent was determined spectrophotometrically in a 0.1 M sodium hydroxide solution [13] with a Zeiss spectrophotometer, model PMQ II. The amino acid analyses were carried out in duplicate, and the average value is reported. Threonine and serine values were corrected for degradation during hydrolysis: threonine, 5%; serine, 10% [14].

Standard assay conditions

Quinolinate phosphoribosyltransferase was assayed as follows: the reaction mixture contained, in a total volume of 0.5 ml: 25 μmol of Tris/maleate/NaOH buffer (pH 6.1); 0.692 μmol of [2,3,7,8-^{14}C]quinolinic acid (0.151 μCi); 1 μmol of *P*-Rib-*P*-*P*, 0.5 μmol of $MgSO_4$ and 10 μg of the crystalline enzyme preparation. After incubating the mixture at 37°C for 30 min, the reaction was stopped by adding 0.8 ml of 4% perchloric acid. The $^{14}CO_2$ evolved, during the shaking of the acidified mixture at 37°C for 90 min, was completely trapped by 0.2 ml of 25% β-phenylethylamine dissolved in methanol on filter paper. The radioactivity was counted with a Packard Tri-Carb liquid scintillation spectrometer, model 2425. Details of the assay procedure were described in a previous paper [15].

Determination of protein

The enzyme concentration was determined spectrophotometrically using an $E^{1\%}_{1\,cm}$ of 9.96 at 280 nm at pH 7.0.

Effect of sulfhydryl reagents and determination of sulfhydryl group

The enzyme protein was previously incubated at 37°C for 30 min with each sulfhydryl reagent in 0.05 M potassium phosphate buffer at pH 7.0. After this incubation, the enzyme activity was assayed by standard assay conditions.

The number of sulfhydryl groups of the enzyme was determined according to the method of Habeeb [16].

Chemical modifications

The enzyme protein (final concentration about 1.2 μM) was incubated with various concentrations of the modifying reagent. Activity was determined by standard assay conditions after modification. As the reference, the same treatment was carried out omitting the modifying reagent.

β-Naphthoquinone-4-sulfonic acid, 2,4,6-trinitrobenzene sulfonic acid, 2-hydroxy-5-nitrobenzyl bromide and acetylimidazole were each dissolved in 0.05 M potassium phosphate buffer, pH 7.5 and the enzyme protein was incubated with each solution at 25°C for 2 h. Diethylpyrocarbonate was dissolved in ethanol, then diluted to various concentrations with 0.1 M potassium phosphate buffer, pH 6.0. Incubation was carried out at 0°C for 1 h. Photooxidation with methylene blue was performed as follows: the enzyme protein was dissolved in 0.02% methylene blue in 0.05 M potassium phosphate buffer, pH 8.0; then oxygen gas was bubbled through this solution for a little while. Irradiation at 25°C was carried out for 2 h with a tungsten lamp (100 W) placed 30 cm above the solution. *p*-Diazobenzenesulfonic acid and glyoxal were dissolved in 0.1 M potassium phosphate buffer, pH 7.5. Incubation was carried out at 25°C for 2 h. Diisopropyl fluorophosphate was dissolved in isopropanol.

Incubation was performed at 25° C for 2 h with the reagent in 0.1 M potassium phosphate buffer, pH 8.0.

Chemical modifications after preincubation with substrates

Chemical modifications were carried out after preliminary incubation of the enzyme with the substrates to obtain information on the substrate binding sites.

Preincubation with the substrates was carried out at 25° C for 15 min. The amount of each substrate and buffer was the same as in the standard assay reaction mixture in a total volume of 100 μl. Final concentrations of substrates and enzyme were as follows: quinolinic acid, 6.92 mM; *P*-Rib-*P*-*P*, 10 mM; Mg^{2+}, 5 mM and the enzyme, 0.588 μM. During this preincubation any enzymic reaction was not occurring because one of substrates (quinolinic acid and *P*-Rib-*P*-*P*) or magnesium ion was omitted from the reaction mixture. After preincubation, the modifying reagent was added and the whole was incubated at 25° C for 1 or 2 h. Enzyme activity was then determined by standard assay conditions.

Results and Discussion

Amino acid composition

The amino acid composition of the enzyme is shown in Table I. It was noted that the alanine value was the highest (213 residues per mol) and the

TABLE I

AMINO ACID COMPOSITION OF THE QUINOLINATE PHOSPHORIBOSYLTRANSFERASE FROM HOG LIVER

The enzyme protein was hydrolyzed at 110°C for 22 h in a 6 M hydrochloric acid. Half cystine and methionine were determined after performic acid oxidation. The tryptophan content was determined spectrophotometrically. Threonine and serine values were corrected for degradation during hydrolysis: threonine, 5%; serine, 10%. The average value of duplicate analyses based on a molecular weight of 172 000, calculated from sedimentation velocity analysis [11], is shown. The yield from amino acid analyses was 97.4%. Ammonia was found to have 116 residues/mol.

Amino acid	(Residues/mol)
Lysine	40
Histidine	26
Arginine	48
Aspartic acid	83
Threonine	71
Serine	62
Glutamic acid	129
Proline	95
Glycine	135
Alanine	213
1/2 Cystine	29
Valine	132
Methionine	18
Isoleucine	14
Leucine	166
Tyrosine	20
Phenylalanine	47
Tryptophan	20

isoleucine value was the lowest (14 residues per mol). The enzyme protein contained 29 residues of half cystine per mol.

Inhibition of sulfhydryl reagents

The crystalline hog liver enzyme was strongly inhibited by sulfhydryl reagents as shown in Table II, while the enzyme from the "Shiitake" mushroom was not [17]. This indicates that the sulfhydryl group plays an important role for the hog liver enzyme activity.

Determination of the sulfhydryl group

The reactive (exposed) sulfhydryl group was determined by using 5,5′-dithiobis(2-nitrobenzoic acid). The reaction proceeded slowly and completed within 2580 min as shown in Fig. 1. The reactive sulfhydryl group was determined to be 10.2 groups per mol of the enzyme. The remaining enzyme activity was also determined. About 50% of the activity was lost when the most reactive single sulfhydryl group was blocked by 5,5′-dithiobis(2-nitrobenzoic acid) (Fig. 1). After this blockage, the enzyme activity gradually decreased. About 20% of the activity remained even when 7 sulfhydryl groups were blocked. The activity was not completely lost during 43 h of incubation with the reagent. The total sulfhydryl group was determined in the presence of sodium dodecyl sulfate. The reaction rapidly proceeded and was completed in 30 min as shown in Fig. 2. The total sulfhydryl group was determined to be

TABLE II

INHIBITION OF QUINOLINATE PHOSPHORIBOSYLTRANSFERASE ACTIVITY BY SULFHYDRYL REAGENTS

The enzyme protein was hydrolyzed at 110°C for 22 h in 6 M hydrochloric acid. Half cystine and indicated at pH 7.0. After incubating the enzyme at 37°C for 30 min, the enzyme activity was determined by standard assay conditions.

SH Reagent	Concentration (M)	Inhibition (%)
p-Chloromercuribenzoic acid	$5 \cdot 10^{-7}$	0.7
	$5 \cdot 10^{-6}$	38.9
	$5 \cdot 10^{-5}$	99.6
	$5 \cdot 10^{-4}$	99.8
N-Ethylmaleimide	$5 \cdot 10^{-5}$	0
	$5 \cdot 10^{-4}$	55.3
	$5 \cdot 10^{-3}$	86.0
	$5 \cdot 10^{-2}$	97.4
5,5′-Dithiobis(2-nitrobenzoic acid)	$1 \cdot 10^{-5}$	1.0
	$1 \cdot 10^{-4}$	16.6
	$1 \cdot 10^{-3}$	45.4
	$1 \cdot 10^{-2}$	88.7
Monoiodoacetic acid	$5 \cdot 10^{-5}$	0
	$5 \cdot 10^{-4}$	11.0
	$5 \cdot 10^{-3}$	50.2
	$5 \cdot 10^{-2}$	99.8

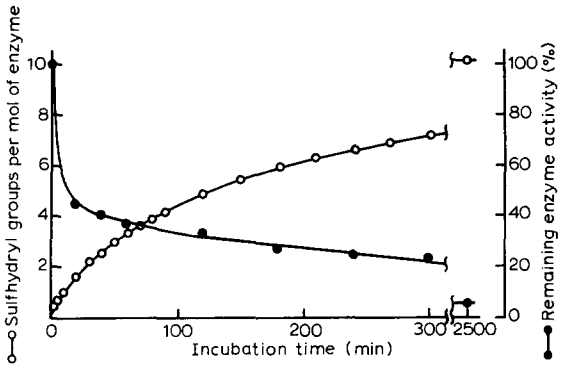

Fig. 1. Loss of quinolinate phosphoribosyltransferase activity as the result of blocking the sulfhydryl group. ○———○, blocked sulfhydryl group; ●———●, remaining enzyme activity. The reaction mixture (3.38 μM of quinolinate phosphoribosyltransferase; 83 mM of sodium phosphate buffer, pH 8.0; 1.64 mM of EDTA; 338 μM of 5,5′-dithiobis (2-nitrobenzoic acid)) was incubated at 25°C for the indicated time. The absorbance at 410 nm was measured with a Hitachi spectrophotometer, model 124 and the enzyme activity was determined by standard assay conditions. A reagent blank was subtracted from the apparent absorbance to give the net absorbance. For calculation of sulfhydryl content, the net absorbance was used with a molar absorption value of 13 600 $M^{-1} \cdot cm^{-1}$.

25.2 groups per mol of the enzyme. Since the molecular weight of the hog liver enzyme was estimated to be 172 000, and that of the subunit to be 34 200 [11], 5 sulfhydryl groups would be present in one subunit of the enzyme and two of them would be the reactive sulfhydryls.

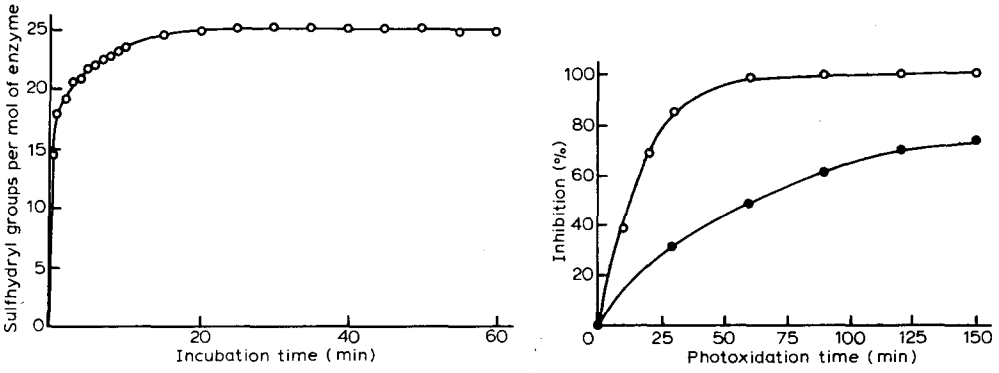

Fig. 2. Determination of the total sulfhydryl group of the quinolinate phosphoribosyltransferase from hog liver by 5,5′-dithiobis(2-nitrobenzoic acid) in the presence of sodium dodecyl sulfate. Conditions were the same as in Fig. 1, except that the dodecyl sulfate was present at a final concentration of 2% in the incubation mixture.

Fig. 3. Protection against the photooxidation of quinolinate phosphoribosyltransferase by preincubation of the enzyme with quinolinic acid. ○———○, without preincubation; ●———●, with preincubation. The preincubation mixture (0.55 μM of quinolinate phosphoribosyltransferase; 50 mM of Tris/maleate/NaOH buffer, pH 6.1; 6.6 mM of [2,3,7,8-^{14}C] quinolinic acid, 1.44 μCi/ml) was incubated at 25°C for 30 min. An equal volume of 0.01% methylene blue in 0.1 M glycine/NaOH buffer, pH 9.0 was added to the preincubation mixture, and the whole was illuminated at 25°C by a 100 W tungsten lamp from a height of 20 cm. An aliquot (190 μl) was withdrawn at the time indicated, and the enzyme activity was determined by the standard assay conditions.

Inhibition by chemical modifying reagents

The effects of chemical modifying reagents on the crystalline hog liver enzyme activity were investigated. The reagents used are known to specifically combine with a certain amino acid as follows: β-naphthoquinone-4-sulfonic acid [18] and 2,4,6-trinitrobenzene sulfonic acid [19] for lysine residue; diethylpyrocarbonate [20], photooxidation in the presence of methylene blue [21] and *p*-diazobenzenesulfonic acid [22] for the histidine residue; glyoxal [23] for the arginine residue; diisopropyl fluorophosphate [24] for the serine residue; 2-hydroxy-5-nitrobenzyl bromide [25] for the tryptophan residue and acetylimidazole [26] for the tyrosine residue. The results, as shown in Table III, indicate that the enzyme activity was extremely inhibited by lysine residue modifying reagents, histidine residue modifying reagents, and an arginine resi-

TABLE III

INHIBITION OF QUINOLINATE PHOSPHORIBOSYLTRANSFERASE ACTIVITY BY VARIOUS CHEMICAL MODIFYING REAGENTS

The enzyme protein (final concentration about 1.2 μM) was incubated with each reagent at the various concentrations indicated. Enzyme activity was then determined by standard assay conditions. Details are described in the text.

Reagent	Concentration (M)	Inhibition (%)
β-Naphthoquinone-4-sulfonic acid	10^{-5}	61
	10^{-4}	98
	10^{-3}	100
2,4,6-Trinitrobenzene sulfonic acid	10^{-5}	27
	10^{-4}	92
	10^{-3}	100
Diethylpyrocarbonate	10^{-5}	4
	10^{-4}	8
	10^{-3}	25
Methylene blue (Photooxidation)	0.02%	94
p-Diazobenzenesulfonic acid	10^{-6}	6
	10^{-5}	55
	10^{-4}	100
Glyoxal	0.04%	34
	0.4%	89
	4%	100
Diisopropyl fluorophosphate	10^{-4}	0
	10^{-3}	0
	10^{-2}	0
2-Hydroxy-5-nitrobenzyl bromide	10^{-5}	2
	10^{-4}	0
	10^{-3}	4
Acetylimidazole	10^{-5}	0
	10^{-4}	0
	10^{-3}	0

TABLE IV

EFFECT OF PREINCUBATION WITH SUBSTRATES ON THE CHEMICAL MODIFICATION OF QUINOLINATE PHOSPHORIBOSYLTRANSFERASE

The preincubation mixture was the same as the standard assay reaction mixture except that the total volume was 100 μl, one or two substrates and Mg^{2+} were omitted as indicated, and incubation was at 25°C for 15 min. The 5,5′-dithiobis(2-nitrobenzoic acid) solution was added at a final concentration of 10 mM and the whole was incubated at 25°C for 2 h at pH 8.0. β-Naphthoquinone-4-sulfonic acid was used at a final concentration of 0.1 mM and incubated at 25°C for 1 h at pH 8.0. *p*-Diazobenzenesulfonic acid was used at a final concentration of 0.1 mM and incubated at 25°C for 1 h at pH 7.5. Enzyme activity was determined by standard assay conditions.

Modifying reagent	Preincubation with			Inhibition (%)
	Quinolinic acid	*P*-Rib-*P*-*P*	$MgSO_4$	
5,5′-Dithiobis(2-nitrobenzoic acid)	—	—	—	93.7
	+	—	—	19.6
	—	+	—	67.0
	—	—	+	86.2
	+	+	—	18.3
	+	—	+	12.3
	—	+	+	27.7
β-Naphthoquinone-4-sulfonic acid	—	—	—	58.7
	+	—	—	5.7
	—	+	—	18.9
	—	—	+	48.1
	+	+	—	3.7
	+	—	+	0
	—	+	+	17.2
p-Diazobenzenesulfonic acid	—	—	—	84.3
	+	—	—	3.1
	—	+	—	35.1
	—	—	+	65.9
	+	+	—	0
	+	—	+	5.1
	—	+	+	38.2

due modifying reagent (glyoxal). But serine residue-, tryptophan residue- and tyrosine residue-modifying reagents had no effect on the enzyme activity.

Effect of preincubation with the substrates on chemical modifications

To get further information on the substrate binding sites of the enzyme protein, the effect of preincubation with substrates on the chemical modifications was investigated. The results are shown in Table IV. It was shown that the inhibition by glyoxal could not be prevented by preincubation with quinolinic acid, whereas the inhibition by β-naphthoquinone-4-sulfonic acid and by *p*-diazobenzenesulfonic acid were completely prevented by preincubation with quinolinic acid (or quinolinic acid and Mg^{2+}). These results suggest that the lysine residue, histidine residue and sulfhydryl group may be closely related to the binding site of quinolinic acid on the enzyme protein molecule.

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