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IDENTIFICATION OF THE CHEMICAL GROUPS INVOLVED IN THE BINDING OF PERIODATE-OXIDIZED NADP$^+$ TO 6-PHOSPHOGLUCONATE DEHYDROGENASE

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Summary

Periodate-oxidized NADP$^+$ binds specifically and reversibly to the NADP$^+$ binding site of 6-phosphogluconate dehydrogenase (EC 1.1.1.44) from *Candida utilis*. The inhibition can be stabilized by reduction with sodium borohydride. It has been shown that an aldehydic group of the inhibitor forms a Schiff base with a lysine residue of the enzyme.

Introduction

In a previous paper [1] we reported that the incubation of 6-phosphogluconate dehydrogenase (EC 1.1.1.44) with periodate-oxidized NADP$^+$ results in the inhibition of the enzyme. The high affinity of the inhibitor for the enzyme, the stoichiometry of the inhibition and the protection by NADP$^+$ indicated that the inhibitor binds specifically to the NADP$^+$ binding site of the enzyme.

In the present paper we report the stabilization, by reduction, of the bond between the inhibitor and the enzyme and the identification of the chemical groups involved in the binding.

Materials and Methods

The 6-phosphogluconate dehydrogenase (6-phospho, D-gluconate: NADP oxidoreductase, decarboxylating, E.C. 1.1.1.44) from *Candida utilis* and the periodate-oxidized NADP$^+$ were prepared as previously described [2,1]. Periodate-oxidized NADP$^+$ was freed from iodate by gel filtration through a column (1.8 × 40 cm) of Sephadex G-10 equilibrated with distilled water.

Tritium labelled NADP$^+$ was prepared by treating the NADP$^+$ (obtained from Sigma Chemical Co., St. Louis, Mo.) first with tritium-labelled sodium boro-

hydride (Radiochemical Center, Amersham) and then with traces of phenazine methosulfate (Sigma); this last product was then removed by absorption on Dowex-50 H^+ resin.

The incubation of the enzyme (3 mg/ml) with periodate-oxidized $NADP^+$ (0.65 μmol/ml) was carried out in 50 mM phosphate buffer, pH 7.5, at room temperature (22° C). The reduction of the enzyme-inhibitor complex was achieved by adding a few crystals of sodium borohydride to the solution of the inhibited enzyme.

Hydrolysis of the reduced enzyme-inhibitor complex

The enzyme-inhibitor complex, after treatment with tritium labelled sodium borohydride, was treated with traces of phenazine methosulfate, to oxidize the reduced nicotinamide ring, precipitated by 80% saturated ammonium sulfate and isolated by gel filtration. The reduced complex was then treated with 1 M NaOH, for 1 h at room temperature, to hydrolyse the *N*-glycosidic bond at the nicotinamide moiety [3] and then dialysed for 2 days against distilled water, until no more radioactivity (due to the radioactive pyridine ring and sugar fragments) was detected in the dialysate. The protein derivative was then hydrolysed in 5.7 M HCl for 24 h at 110° C. Half of the hydrolysate was treated with periodate.

Preparation of the standards for the chromatography

The scheme of the pathway followed in the preparation of the standards is shown in Fig. 1. α-*N*-Acetyllysine was incubated with D-glyceraldehyde (Sigma) at pH 7.5 for 20 min; the Schiff base formed was reduced with tritium labelled sodium borohydride. The reduced compound was treated with HCl, as above, to hydrolyse the N-acetyl bond. The hydrolysate, which contains compound I of Fig. 1, was divided in two parts, one of which was treated with periodate to obtain compound II. The two samples (treated and untreated with periodate) were purified by column chromatography on Dowex 50 H^+ resin, which retains the lysine and lysine derivatives. These compounds were eluted with 0.2 M NH_4OH. Each eluate, on thin layer chromatography, gave only two ninhydrin positive spots, one of which was radioactive. The other was non-radioactive and had the same R_F value as authentic lysine. The radioactive, ninhydrin-positive samples were taken as standards for subsequent chromatography.

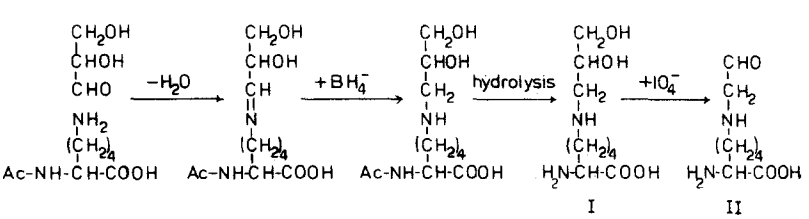

Fig. 1. Scheme of the preparation of standards for thin layer chromatography.

Thin layer chromatography

Thin layer chromatography was carried out on glass plates covered with a layer (250 μm thick) of Silica gel (type 60, Merck) in two solvents. Solvent A was composed of 1-propanol/25% ammonia (67 : 33, v/v), while solvent B was *n*-butanol/acetic acid/water (60 : 20 : 20). After chromatography, the plates were dried and sprayed with ninhydrin. Marks were made every 0.5 cm and the gel of these sections was removed, suspended in Bray [4] solution and counted in a Packard liquid scintillation counter.

Results

Stabilization of the enzyme inhibitor complex

Using gel filtration and periodate-oxidized $NADP^+$ labelled with tritium on the nicotinamide ring, it is possible to study the stoichiometry and the reversibility of the inhibition.

The enzyme, after incubation with a 10-fold molar excess of radioactive periodate-oxidized $NADP^+$, was 8% active (Table I, B) as compared with the native enzyme. The enzyme-inhibitor complex was passed twice through a column (0.80 × 40 cm) of Sephadex G-25 equilibrated with 50 mM phosphate buffer, pH 7.5, and the fractions were immediately analysed for protein concentration, enzymatic activity and radioactivity (Table I, C and D). As can be seen from the data reported in Table I, after each gel filtration there was a decrease of inhibitor bound to the enzyme and a recovery of activity, while the ratio [inhibitor]/[inactive subunit] was constant and near unity.

If, instead, the enzyme-inhibitor complex was pretreated with borohydride, there was no recovery of activity, either by dilution or by gel filtration (Table I, E) and after gel filtration the ratio [inhibitor]/[inactive subunit] was still near unity.

These results confirm the reversibility and the 1 : 1 stoichiometry of the inhibition [1]. Furthermore, they indicate that the binding between the inhibitor and the enzyme can be stabilized by reduction, thus suggesting the involvement

TABLE I

GEL FILTRATION OF THE ENZYME-INHIBITOR COMPLEX

The enzyme was treated with periodate-oxidized $NADP^+$ at pH 7.5 (details in the text) until 92% inhibition was reached, line B). The sample was divided in two parts: one was subjected to two consecutive gel filtrations (C and D), the other was first treated with sodium borohydride and then subjected to gel filtration (E). The fractions after gel chromatography were immediately analysed for enzyme activity, protein concentration and radioactivity.

	Step	[subunit] (μM)	[inhibitor] (μM)	Enzymatic activity (%)	[Inactive subunit] (μM)	[Inhibitor] / [Inactive subunit]
A	Untreated enzyme	60	0	100	—	—
B	Inhibited enzyme	60	605	8	56	11.7
C	After first gel filtration	30	19.4	32	20.4	0.95
D	After second gel filtration	13	3.5	70	3.9	0.9
E	After reduction and gel filtration	32	28	10	29	0.97

of a Schiff base, formed between one of the aldehydic groups of the inhibitor and a lysine residue of the enzyme, in the binding.

Identification of the chemical groups involved in the binding of the inhibitor to the enzyme

The treatment of $NADP^+$ with periodate results in the cleavage of the bond between carbons 2′ and 3′ of the ribose ring bound to nicotinamide (Fig. 2) and the formation of two aldehydic groups at these carbons. Both aldehydic groups could form a Schiff base with a lysine residue of the enzyme. After reduction with borohydride and hydrolysis (of the N-glycosidic bond at the nicotinamide, of the ester bond at the carbon 5′ of the former ribose ring, and of the peptide bonds of the protein) compounds I and II could be formed (Fig. 2), the first being convertible into the second by periodate oxidation. These two compounds were chemically synthesized as indicated in Fig. 1.

The enzyme was treated with non radioactive periodate-oxidized $NADP^+$ and radioactive borohydride and then hydrolysed. The chromatography of the hydrolysate in solvent A (see Materials and Methods) yielded two almost equivalent radioactive peaks (Fig. 3, bottom, broken line), with the same R_F values of the two standards. If the hydrolysate was pretreated with periodate, the chromatography revealed the disappearance of compound I and its complete transformation into compound II (Fig. 3, bottom, solid line).

The same results, although with a lower resolution, were obtained with solvent B. The R_F values (lower than 1.5) of the radioactive peaks, in this solvent, indicate that the radioactive compounds have a strong basic character; this rules out the involvement of amino acids other than lysine and arginine in the binding of the inhibitor. Our attempts to obtain a reduced Schiff base between the guanidino group of arginine and glyceraldehyde failed; and, to the best of our

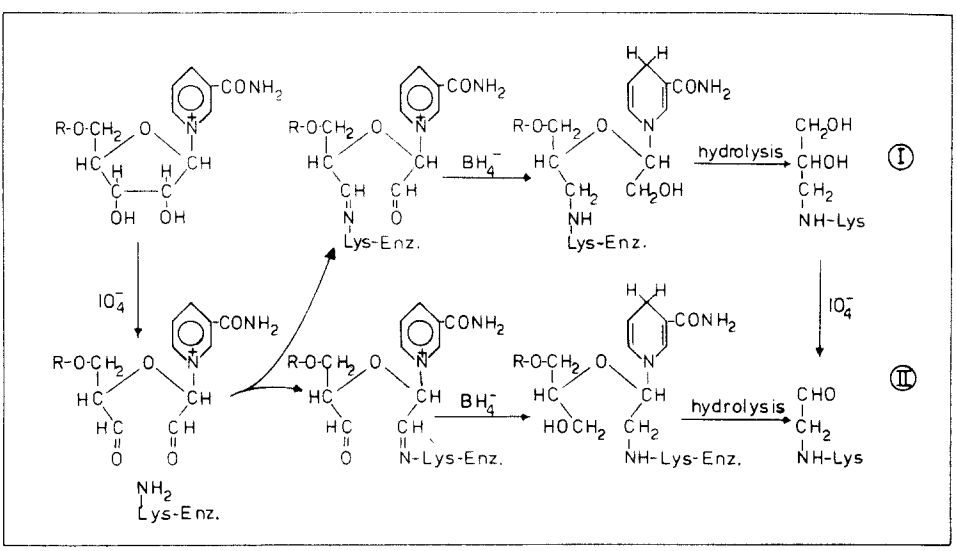

Fig. 2. Pathway followed for the identification of the amino acid residue involved in the binding of periodate-oxidized $NADP^+$ to the enzyme.

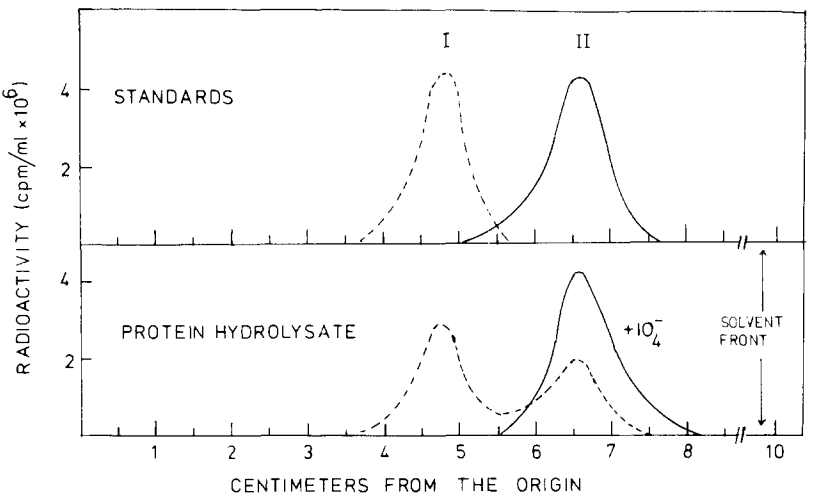

Fig. 3. Analysis of the products of hydrolysis of reduced enzyme-inhibitor complex on thin layer chromatography in solvent A (see Materials and Methods).

knowledge, the formation of such a Schiff base was never reported. On the basis of these results, we believe that an aldehydic group of the inhibitor forms a reducible Schiff base with a lysine residue of the enzyme.

Discussion

The periodate-oxidized $NADP^+$ binds specifically and reversibly to the $NADP^+$ binding site of 6-phosphogluconate dehydrogenase, causing the inhibition of the enzyme [1]. This inhibition can be made irreversible by treatment of the enzyme-inhibitor complex with sodium borohydride, which reduces the Schiff base formed between a lysine residue of the enzyme and one of the two aldehydic groups of the inhibitor.

Upon complete hydrolysis, a lysine residue has been found bound to either a two or a three carbon chain, thus indicating that both aldehydic groups of the inhibitor can form a Schiff base with the lysine residue.

We are tempted to hypothesize that the same residue of lysine is involved into the two cases, thus implying that the two aldehydic groups of the inhibitor are very close each to the other, when the inhibitor is bound to the enzyme. This hypothesis can be confirmed only upon the determination of the amino acid sequence around the lysine residue.

The possibility of transforming the aldehydic groups of the inhibitor into other chemically reactive groups and the induction of irreversible binding of the inhibitor to the enzyme by reduction, make the periodate-oxidized ribotides a new promising kind of inhibitor which could be useful for the elucidation of the structure and functions of proteins which use ribotides as substrate, coenzyme or effector.

Acknowledgements

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