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PURINE NUCLEOSIDE PHOSPHORYLASE: IMMOBILIZATION BY COVALENT CHROMATOGRAPHY AND A STUDY ON ITS SULFHYDRYL GROUPS

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Covalent chromatography is used for studying the role of sulfhydryl groups in pig brain purine nucleoside phosphorylase (PNP). This enzyme has been immobilized on an insoluble polymeric reagent (thiol-Sepharose 4B) by a thiol-disulfide interchange reaction between the disulfide groups of the gel and some of its thiol groups. The immobilized enzyme retained its activity and the coupling was reversible under reducing conditions, allowing the recovery of the enzymic activity. These results suggest that PNP contains nonessential sulfhydryl groups that can react with the thiol-Sepharose. On the other hand, inactivation with some thiol reagents shows that thiol groups directly involved in the catalytic activity are present at or near the active site. The technique described should be generally useful in the immobilization of thiol-containing proteins and in the characterization of these thiol groups.

INTRODUCTION

Purine nucleoside phosphorylase (PNP) catalyzes both the synthesis and degradation of purine nucleosides and may be involved in the control of intracellular levels of nucleic acids. The enzyme is largely distributed in mammalian tissues (1–7) and is highly active in the cytoplasmic supernatant fraction from pig brain, where a large concentration of guanine deaminase was previously reported by this laboratory (8). These enzymes play a strictly related role in purine metabolism. PNP has been widely studied; data on the presence and the role of thiol groups in the enzyme from rabbit liver, bovine brain, and human erythrocytes have been reported (3,4,9).

In an earlier communication, we described the immobilization of guanine deaminase by covalent chromatography and made observations on the role of its cysteine residues (10). The present paper is concerned with a method for covalent reversible binding of PNP to a mixed disulfide derivative carried by a Sepharose matrix. The activity is still shown by the immobilized enzyme: this indicates the existence of thiol groups that are not

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involved in the catalysis. On the other hand, the observation that sulfhydryl reagents such as *p*-chloro-mercuribenzoate (PCMB) and 5,5′-dithiobis(2-nitro)benzoic acid (DTNB) inactivate the enzyme, and the recovery of the activity by addition of an excess of 2-mercaptoethanol, suggests the presence of one or more essential —SH groups. The reversible coupling of PNP to the insoluble polymeric reagent suggests the general usefulness of this reagent in studying the presence of nonessential —SH residues.

MATERIALS AND METHODS

Materials

Unless otherwise stated, all chemicals were of analytical reagent grade. Fresh pig brain was from a commercial slaughterhouse. Activated thiol-Sepharose 4B was purchased from Pharmacia Fine Chemicals. Iodoacetamide, 2-mercaptoethanol, DTNB, and PCMB were from Eastman Kodak. Guanine and inosine were purchased from Boehringer. Xanthine oxidase was purified from buttermilk following Massey et al. (11).

Assay for Purine Nucleoside Phosphorylase Activity

The assay is a modification of the coupled xanthine oxidase method of Kalckar (12), which is based on the measurement of the absorbance at 293 nm due to the formation of uric acid. The reaction mixture contained inosine, 0.75 μmol, sodium phosphate buffer, pH 7.4, 50 μmol, xanthine oxidase, 0.05 unit, and an appropriate amount of PNP, in a cuvette having a 1 cm length path, in a final volume of 1 ml, at room temperature.

One unit of PNP is defined as the amount of enzyme that gives an optical density increase of 12.5 per min (1 μmol of uric acid formation) under standard assay conditions.

Protein Determination

Protein concentration was determined spectrophotometrically by measuring the optical density at 280 and 260 nm and using the equation employed by Kalckar (13), or by the method of Lowry et al. (14), with bovine serum albumin as standard.

Enzyme Preparation

The enzyme was extracted from pig brain with 2 vol cold 20 mM phosphate buffer, pH 7.0, and partially purified by ammonium sulphate

fractionation and DEAE-cellulose chromatography to a specific activity of 0.1 unit per mg of protein.

Response to Thiol Reagents

The studies on inactivation by PCMB, DTNB, and iodoacetamide were performed by incubating the enzyme with 0.06 mM PCMB, 0.1 mM DTNB, and 0.4 mM iodoacetamide, respectively. The buffer used was 100 mM tris-acetate, pH 7.9, for PCMB and DTNB, while 50 mM phosphate buffer, pH 7.2, was used for the reaction with iodoacetamide.

Coupling of Purine Nucleoside Phosphorylase

A column with a total volume of 20 ml (1.6 × 10 cm) was prepared by pouring a suspension of activated thiol-Sepharose 4B into a chromatographic tube. The gel was equilibrated with 20 mM phosphate buffer, pH 7, containing 1 mM EDTA and 0.3 M NaCl. The enzyme was dialyzed against the same buffer and passed through the column at a flow rate of 10 ml/h. The reaction of PNP with the mixed-disulfide residues of the gel was followed by monitoring the 2-thiopyridone released at 343 nm (15,16).

The gel was then washed with the same buffer until the absorbance at both 280 and 343 nm decreased to the baseline. The bound enzyme was released, eluting with a cysteine linear gradient (0–30 mM in 0.1 M tris-HCl, pH 8, containing 1 mM EDTA and 0.3 M NaCl; total volume 100 ml).

Immobilized Enzyme Activity Measurement

In order to check the activity of the immobilized PNP, 50 μl of enzymatic solution containing 5 mU was added to 1 ml of thiolated Sepharose. The coupling reaction was carried out batchwise for 1 h, in order to bind all the enzyme to the gel. After washing, the immobilized enzyme was mixed with 10 ml of 0.75 mM inosine in 50 mM phosphate buffer, pH 7.5, and the reaction was allowed to proceed with end-over-end rotation. At intervals, 1.5 ml aliquots of the reaction mixture were removed and rapidly centrifuged. Then 0.8 ml of supernatant was assayed for hypoxanthine with xanthine oxidase at 293 nm. A similar reaction was carried out as a control, incubating in the same reaction mixture 50 μl of an equivalent amount of "free" PNP.

RESULTS

Experiments were carried out with an enzyme preparation from pig brain, with a specific activity of 0.1 U/mg of protein. Covalent chromatography allows one to couple the enzyme to the mixed disulfide derivative of

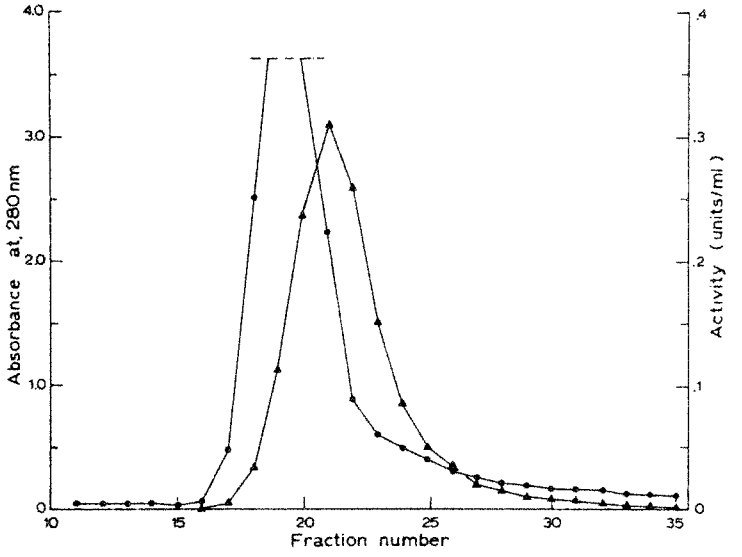

FIG. 1. Elution pattern of purine nucleoside phosphorylase from a thiol-Sepharose 4B column. Enzyme activity (▲—▲) was measured by the coupled xanthine oxidase method. The protein content is indicated by absorbance at 280 nm (●—●). Other details are described in the text under Methods.

Sepharose and to separate it from proteins with only low reactivity toward these disulfide groups (15). The purine nucleoside phosphorylase bound to thiol-Sepharose retained an activity equivalent to 80% of the free enzyme when measured as described above. The enzyme can be totally recovered with cysteine, while the use of a linear gradient elution also accomplishes a partial purification (three to five fold). The elution profile is given in Fig. 1.

The partial selectivity and the specificity of the thiolated gel might result from the high content of "free" —SH groups of this protein. The activity of the immobilized enzyme suggests that thiol groups reacting with the disulfide derivative are not important for the catalytic activity. These findings led us to investigate the reactivity of PNP toward a variety of thiol reagents.

When the enzyme was incubated at room temperature for 15 min with different concentrations of PCMB, almost complete inactivation occurred at 1×10^{-5} M PCMB, as shown in Fig. 2. The inactivation by PCMB at concentrations as high as 1×10^{-4} M could be completely reversed by the addition of an excess of 2-mercaptoethanol. The enzyme, which was rapidly inactivated by PCMB, was unresponsive to inactivation by iodoacetamide at

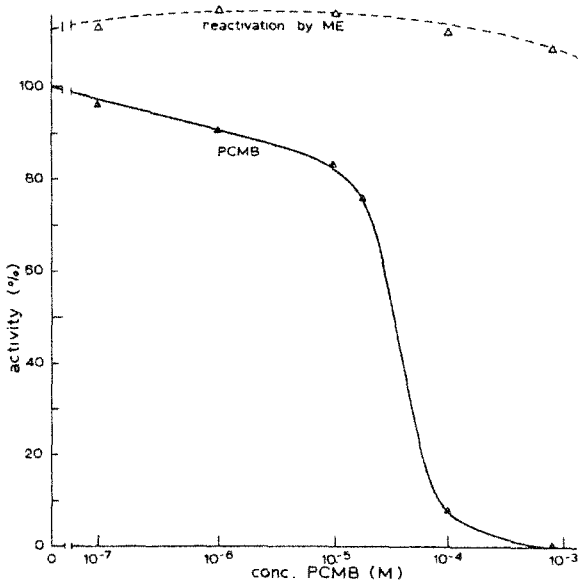

FIG. 2. Effect of PCMB concentration on purine nucleoside activity. Reaction mixtures in a volume of 0.5 ml contained 0.1 ml of PNP preparation (0.05 U), 100 mM tris-acetate, pH 7.9, and PCMB at varying concentrations. After incubation for 10 min at room temperature, 50 μl aliquots were withdrawn and assayed for enzymic activity after 20-fold dilution. Twenty mM 2-mercaptoethanol (ME) were added to the remaining solutions, and after 3 h of incubation at room temperature, enzymic activity was assayed.

concentrations of 0.4 mM (Fig. 3). PNP was highly susceptible even to inactivation by 0.1 mM DTNB.

In order to control whether a substrate such as guanine has any effect on the reactivity of sulfhydryl groups with DTNB, we added 0.1 mM guanine to the incubation mixture. Figure 4 shows the rate of inactivation in the presence and absence of guanine.

Reaction with DTNB caused a nearly immediate decrease in enzymic activity of 60%, whereas the loss of activity in the presence of guanine was slower. Furthermore, when reactivation was attempted by addition of an excess of 2-mercaptoethanol, rapid and complete restoration of activity occurred, whereas in the absence of guanine, only partial restoration (about 80%) was possible. This suggests that the tertiary structure of the protein may explain why extra —SH groups react with DTNB in the absence of guanine, and when this happens, irreversible denaturation can occur.

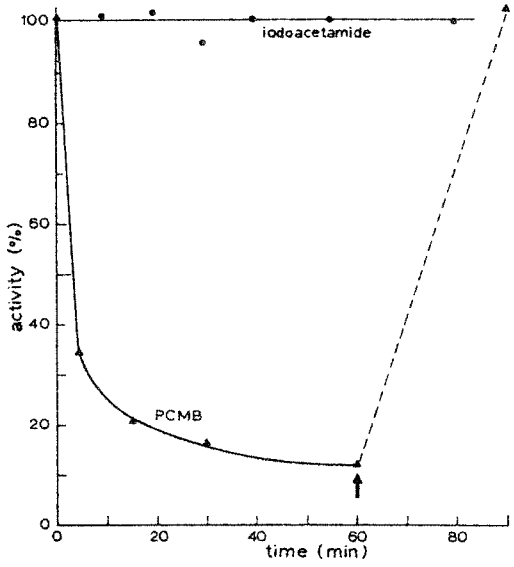

FIG. 3. Rate of inactivation of PNP with PCMB and iodoacetamide. Reaction mixture A made up 0.5 ml, 50 mM phosphate buffer and 400 mM iodoacetamide (●—●). Reaction mixture B made up the same volume, 100 mM tris-acetate buffer and 0.06 mM PCMB (▲—▲). Both were incubated at room temperature with 0.05 U of PNP. Aliquots were withdrawn at various intervals and assayed for enzymic activity. After 60 min, 20 mM 2-mercaptoethanol was added to a fraction of mixture B, as indicated by the arrow, and enzymic activity was measured after 30 min of incubation at room temperature, as shown by the dashed lines.

DISCUSSION

Results of this study strongly show an important role for —SH groups in the activity of porcine PNP and suggest that different types of sulfhydryl residues exist in this enzyme. In fact, purine nucleoside phosphorylase is rapidly coupled to the mixed disulfide derivative of Sepharose. The immobilized enzyme retains its activity, demonstrating the presence of sulfhydryl groups, which do not appear to be directly involved in enzymic activity. In crude enzyme preparations, a similar conclusion could not be obtained by the usual thiol reagents. The specificity of the coupling reaction and the recovery of the enzymatic activity by elution with cysteine show that only —SH groups are involved in the complex formation.

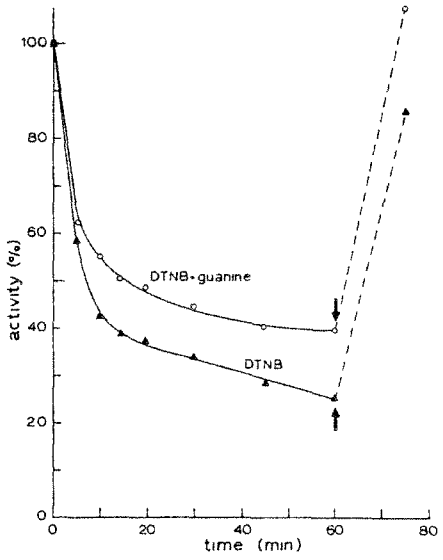

FIG. 4. Rate of inactivation of PNP with DTNB. Two incubation mixtures made up 0.5 ml; 100 mM tris-acetate, pH 7.9; purine nucleoside phosphorylase, 0.05 U; 0.1 mM DTNB. To one reaction mixture was added guanine, 0.1 mM, before the addition of DTNB. ○—○, plus guanine; ▲—▲, minus guanine. Aliquots were withdrawn at different times and assayed for enzymic activity. After 120 min of incubation, to a fraction of each mixture was added 20 mM 2-mercaptoethanol, as indicated by the arrow, and the enzymic activity was measured after 15 min of incubation at room temperature, as shown by dashed lines.

On the other hand, the complete reactivation of the PCMB-inactivated enzyme with 2-mercaptoethanol suggests a reversible interaction of this thiol reagent with essential cysteine residues at the catalytic site of the enzyme, in agreement with the data of Agarwal and Parks for human erythrocytes (9). Moreover, as seen in Fig. 4, PNP is partially protected from reaction with DTNB when incubated with a purine substrate such as guanine. These findings suggest that a conformational change is induced when the enzyme interacts with guanine, which diminishes the access of DTNB to sulfhydryl groups in an active site of the molecule. The difference in reactivity to PCMB and DTNB might be explained by the difference in charge and size of the two reagents.

The thiol-Sepharose column exhibits high selectivity for binding PNP, which is eluted later than the main part of the proteins of the enzyme preparation. This allows a concomitant partial purification of the enzyme. The complex is stable at least for one month if stored at 4°C, and this gel can be used to concentrate and store diluted enzymatic solutions.

The technique described for immobilizing PNP should be generally applicable for immobilization of thiol-containing enzymes and other thiol compounds and should be useful in the characterization of these thiol groups.

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