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Reaction Mechanism of Purine Nucleoside Phosphorylase and Effects of Reactive Agents for SH Group on the Enzyme in *Saccharomyces cerevisiae*

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Kinetic analysis was done to elucidate the reaction mechanism of purine nucleoside phosphorylase (PNP) in *Saccharomyces cerevisiae*. The binary complexes of PNP · phosphate and PNP · ribose 1-phosphate were involved in the reaction mechanism. The initial velocity and product inhibition studies demonstrated were consistent with the predominant mechanism of the reaction being an ordered bi, bi reaction. The phosphate bound to the enzyme first, followed by nucleoside and base were the first product to leave, followed by ribose 1-phosphate. The kinetically suggested mechanism of PNP in *S. cerevisiae* was in agreement with the results of protection studies against the inactivation of the enzyme by sulfhydryl reagents, p-chloromercuribenzoate (PCMB) and 5,5′-dithiobisnitrobenzoate (DTNB). PNP was protected by ribose 1-phosphate and phosphate, but not by nucleoside or base, supporting the reaction order of ordered bi, bi mechanism. PCMB or DTNB-inactivated PNP was totally reactivated by dithiothreitol (DTT) and the activity was returned to the level of 77% by 2-mercaptoethanol, indicating that inactivation was reversible. The kinetic behavior of the PCMB-inactivated enzyme had been changed with higher K_m value of inosine and lower V_m, and was restored by DTT. Inactivation of enzyme by DTNB showed similar pattern of K_m value with that by PCMB, but had not changed the V_m value, significantly. Negative cooperativity was not found with PCMB or DTNB treated PNP at high concentration of phosphate.

KEY WORDS □ **purine nucleoside phosphorylase, *S. cerevisiae*, reaction mechanism, PCMB, DTNB**

Purine nucleoside phosphorylase (PNP) is a key enzyme in the purine salvage pathway. It catalyzes reversibly the phosphorolysis of purine nucleosides and deoxynucleosides according to the following reactions:

guanosine + Pi ⟷ guanine + ribose 1-phosphate
inosine + Pi ⟷ hypoxanthine + ribose 1-phosphate

At equilibrium, the direction of nucleoside synthesis is favored, but net flux of the reaction in intact cells is in the catabolic direction. The reactions catalyzed by this enzyme generated the major source of xanthine and hypoxanthine produced in the cells.

The association of a deficiency in PNP with an immunodeficiency disorder has been reported (24, 27, 30). Patients with a genetic deficiency of PNP gene showed severe impairment in T lymphocyte functions, but not in B lymphocyte functions. T cell selective immunosuppressive agents could be used for the treatment of human T cell leukemia and autoimmune diseases and the prevention of organ transplant rejection. A potent PNP inhibitor (8, 11, 16, 34, 36) was considered to cause similar selective inhibition of T cell-mediated immunity. The importance of PNP in immunodevelopment (29, 30) and in the metabolism of purine nucleoside has prompted detailed structural and kinetic studies of the enzyme in mammalian cells (2, 7, 9, 10, 25, 26, 35). However, the utilization of PNP for the enzymatic production of purine nucleoside analogs (38), such as adenine arabinoside for an antiviral drug, has encouraged to screen the bacteria which is a potent producer of PNP (20, 21) and require specific characteristics and kinetic studies of the enzyme in microorganisms.

In a previous paper (5), PNP from *Saccharomyces cerevisiae* has been partially purified and characterized. Kinetic parameters of nucleoside substrates and inhibitors were measured and the phenomena of negative cooperativity for cosubstrate, inorganic phosphate was demonstrated in a following study (6). We have undertaken a detailed kinetic investigation to determine the reaction sequence of PNP in *S. cerevisiae*. In

addition, the effects of sulfhydryl group reagents, p-chloromercuribenzoate (PCMB) and 5,5′-dithiobisnitrobenzoate (DTNB) and thiols, dithiothreitol (DTT) and 2-mercaptoethanol on PNP were determined to understand the role of sulfhydryl group in the enzyme.

MATERIALS AND METHODS

Enzyme purification

Baker's yeast commercially available from Sigma Chemical Co. was used and all purification procedures were followed as described in the previous paper (5). The PNP preparations were used with the specific activities of 0.070~0.080 U/mg. Inosine, deoxyinosine, guanosine, deoxyguanosine, guanine, adenosine, deoxyadenosine, ribose 1-phosphate, xanthine oxidase, DTT, 2-mercaptoethanol, PCMB and DTNB were purchased from Sigma Chemical Co.

Purine nucleoside phosphorylase assay

The phosphorolysis of inosine was measured by a spectrophotometric assay coupled to xanthine oxidase (14). The assay was based on the measurement of the increase in absorbance at 293 nm due to the formation of uric acid ($\Delta\varepsilon=12.5\times10^{-3}$ M^{-1} cm^{-1}). Spectrophotometer (Kontron, UVKON 860) equipped with RS 232C-interface was used. For the kinetic analysis, the reaction mixture contained, in a final volume of 1 ml, 50 mM Tris-Cl, pH 7.3, 100 mM phosphate, 0.02 units of xanthine oxidase, the various concentrations of inosine and an appropriate amount of PNP. All reaction mixture except PNP were preincubated at 30°C for about 3 min to remove any trace of hypoxanthine or xanthine as a contaminant in the inosine. The reaction was started by the addition of PNP and continued for about 3 min to get the linear region. When the concentrations of phosphate were variable, the reaction mixture was the same as above except 1 mM of inosine. The reaction mixture for inhibition studies contained, in a final volume of 1 ml, 50 mM Tris, pH 7.3, 1 mM inosine, 100 mM potassium phosphate, 0.02 μM unit of xanthine oxidase and an appropriate amounts of PNP treated with the indicated concentration of sulfhydryl group reagents. For the substrate protection studies, the enzyme was preincubated with the indicated concentration of each substrate for 10 min and treated with 10 μM PCMB or 77 μM DTNB for 10 min.

Data processing

Points on the kinetic curves represented aver age of two or three determinations, however, in the calculations each determination was treated individually. Reciprocal of velocities were plotted graphically versus reciprocal of substrate concentrations.

$$1/v=K_m/V_m\cdot 1/S+1/V_m$$

The K_i values of inhibitors were calculated from the replots of slopes versus the concentrations of inhibitors shown as insets of figures.

RESULTS

Initial velocity studies

We can divide two substrates reaction such as PNP into two main catagories. One is those involving a ternary complex and the other is a ping-pong mechanism. To distinguish between the various types of possible reaction mechanism, the initial rate of reaction was determined. When inosine was the variable substrate with phosphate as the changing fixed substrate, the Lineweaver-Burk plots reavealed a crossing pattern in which both the slopes and the intercepts changed as shown in Fig. 1. When phosphate was the variable substrate and inosine was the changing fixed substrate, similar phenomena were observed (data not shown). These data excluded the possibility of a ping-pong mechanism. The kinetic parameters were estimated from the primary and secondary plots shown in Table 1.

Product inhibition studies

Product inhibition studies were performed to distinguish between an ordered and a random ternary complex mechanism. When inosine was the various substrate with phosphate of constant saturating level, guanine, the alternative product, was examined as an inhibitor. Guanine showed the noncompetitive inhibition as shown in Fig. 2-A. The inhibition constant for guanine was 5.0×10^{-6} M from the replot of Fig. 2-A. When the ribose 1-phosphate was used as the product inhibitor, no inhibition was found. However, when the concentration of phosphate decreased to be unsaturating, ribose 1-phosphate acted as a noncompetitive inhibitor as shown in Fig. 2-B. With phosphate as the variable substrate at a constant level of inosine, inhibition by guanine was measured. As shown in Fig. 2-C, a pattern of noncompetitive inhibition was observed with the

Table 1. *Kinetic parameters of initial velocity studies.*

Parameter	Phosphorolysis (M)
Inosine, Ka	2.5×10^{-4}
Inosine, Kia	3.1×10^{-4}
Phosphate, Kb	1.6×10^{-3}

The kinetic parameters were calculated by the Lineweaver-Burk plots or the replots of the slopes or the intercepts shown in Fig. 1. The average is given when there is more than one value.

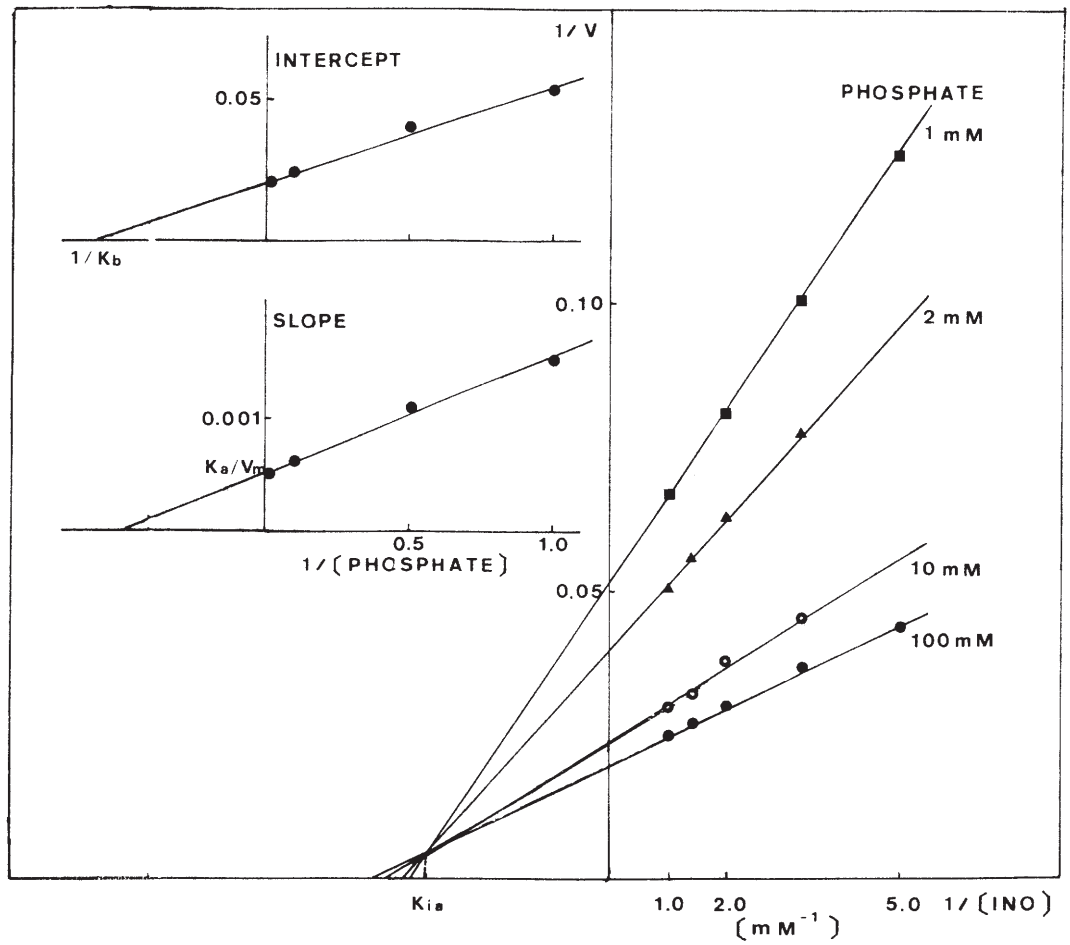

Fig. 1. *The initial velocity study with inosine as the variable substrate and the plot of reciprocal velocity with respective to reciprocal of inosine concentrations.*
The reaction mixture contained in a 1 ml cuvette, 50 mM Tris-Cl, pH 7.3, 0.02 units of xanthine oxidase, indicated amounts of inosine and phosphate. Kinetic parameters are estimated from the plot and replot. Ka, 2.5×10^{-4} M; Kia, 3.1×10^{-4} M; Kb, 1.6×10^{-3} M.

inhibition constant for guanine of 5.7×10^{-6} M. When ribose 1-phosphate was tested as a product inhibitor, ribose 1-phosphate showed the competitive inhibition pattern with the inhibition constant of 6.3×10^{-4} M (Fig. 2-D). It suggested that phosphate and ribose 1-phosphate compete for the same binding site of the enzyme. The patterns of the inhibition from product inhibition studies are shown in Table 2.

Inactivation of purine nucleoside phosphorylase by sulfhydryl reagents

When the enzyme was incubated at room temperature with various concentrations of p-chloromercuribenzoate (PCMB) for the indicated time intervals, no further change of inactivation occurred after about 5 min. The plateau level of inactivation by 5,5′-dithiobisnitrobenzoate (DTNB) occurred at about 10 min of incubation (results not shown). In Order to show the effect of concentration, PNP was incubated for 10 min with the various concentrations of PCMB and DTNB. Total inactivation occurred at 5×10^{-5} M PCMB and 2×10^{-4} M DTNB, as shown in Fig. 3.

Reactivation of PNP by DTT and 2-mercaptoethanol

When the enzyme was totally inactivated by sulfhydryl reagents, the effect of thiols on PNP had been observed. As shown in Fig. 4, PNP was incubated with 10^{-4} M PCMB for 10 min and complete inactivation occurred. The enzyme was

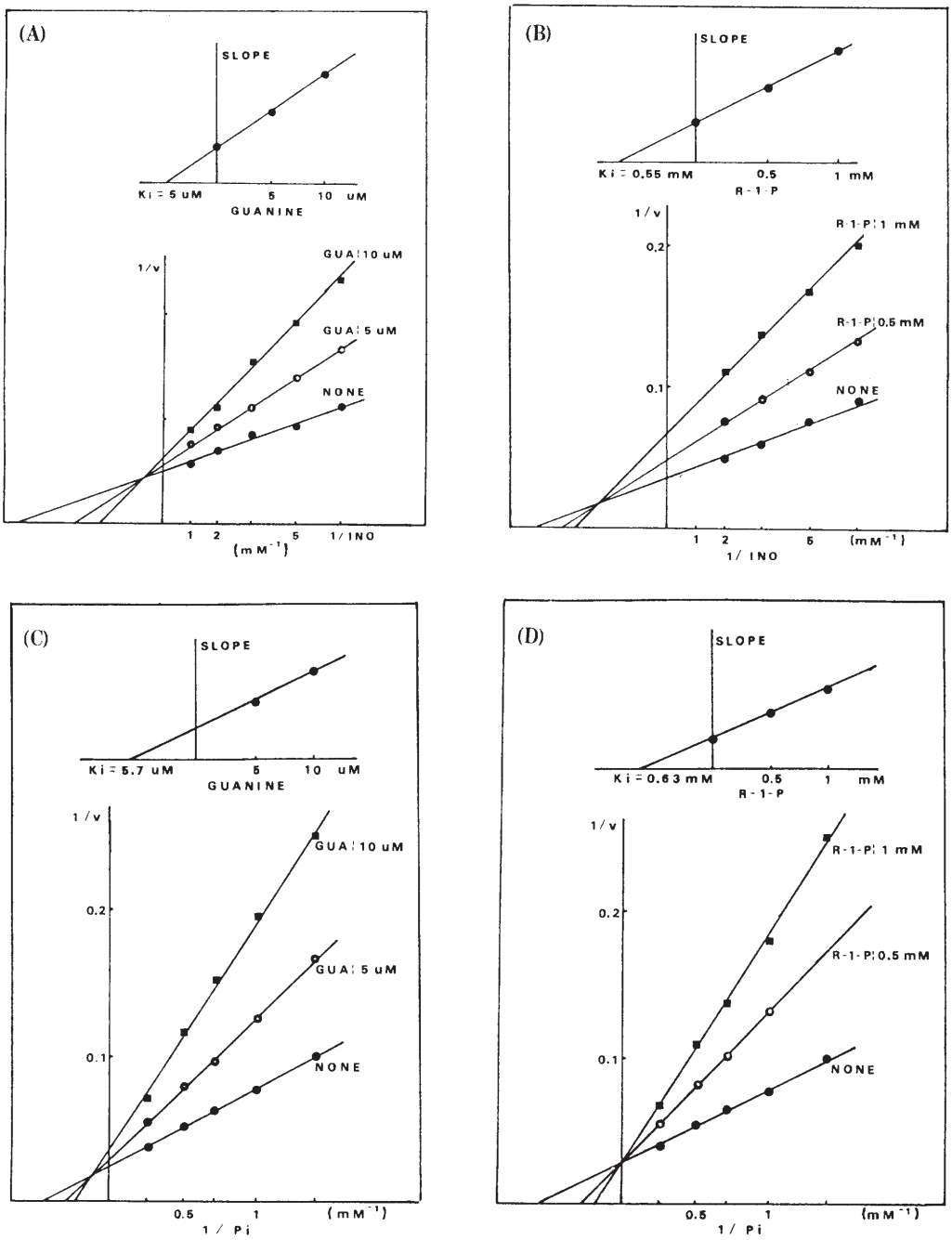

Fig. 2. *Inhibition study with alternative products, guanine (A, C) or ribose 1-phosphate (B, D).*
Plot of reciprocal of initial velocity with respective to reciprocal of inosine (A, B) or phosphate (C, D) concentrations. The reaction mixture contained, in a final volume of 1 m*l*, 50 mM Tris-Cl, pH 7.3, 0.02 units of xanthine oxidase, 100 mM phosphate, the indicated amounts of inosine and alternative product, guanine (A). The reaction mixture was the same as above except 1.5 mM phosphate for alternative product, ribose 1-phosphate (B). When the phosphate concentration was varied, the reaction mixture was the same as above except 1 mM of inosine and indicated amounts of guanine (C) or ribose 1-phosphate (D).

Table 2. *Inhibition patterns of product inhibition studies. The results were from Fig. 2.*

Variable substrate	Product inhibitor	
	Guanine	Ribose 1-phosphate
Inosine	Noncompetitive	No inhibition at 100 mM Pi Noncompetitive at 1.5 mM Pi
Phosphate	Noncompetitive at 1 mM inosine	Competitive at 1 mM inosine

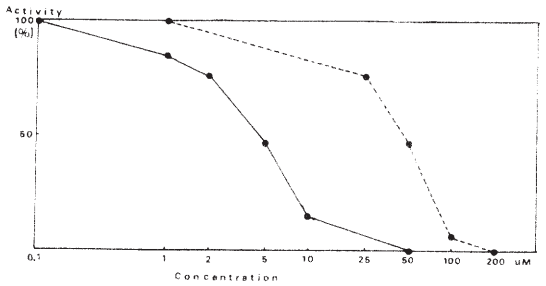

Fig. 3. *Inhibition of purine nucleoside phosphorylase (PNP) by sulfhydryl reagents, PCMB (——) or DTNB (- - - -).*
One milliliter of the reaction mixture contai ned 50 mM Tris-Cl, pH 7.3, 1 mM inosine, 1 00 mM phosphate, 0.02 units of xanthine oxidase and an appropriate amounts of PNP treated with the indicated concentration of PCMB (——) DTNB (- - - -) for 10 min.

reactivated up to 77% after 1 min incubation with an excess of dithiothreitol (DTT) and fully gained the activity within 30 min, showing that PNP was not irreversibly inactivated by PCMB. When the concentration of 10 mM 2-mercaptoethanol was used for reactivation, the activity of PNP returned to the level of 77% and no further reactivation occurred. Fig. 5 showed the effects of DTT and 2-mercaptoethanol on activity of PNP inhibited by 2.5×10^{-4} M DTNB. Similar pattern was observed when the enzyme was reactivated by DTT. However, PNP inhibited by DTNB was reactivated only partially (33%) by the treatment of an excess of 2-mercaptoethanol. The quantitative dif-fference in the effects of thiols, 2-mercaptoethanol and DTT, on the enzyme was more higher with PNP inhibited by DTNB than by PCMB. It seemed that those sulfhydryl reagents, PCMB or DTNB, and thiols, DTT or 2-mercaptoethanol,
reacted with different parts of sulfhydryl groups

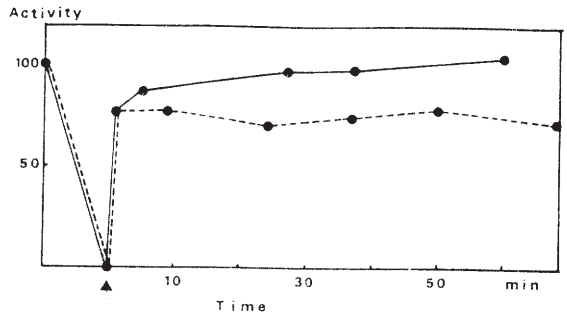

Fig. 4. *Reactivation of PCMB treated PNP by thiol reagents, DTT (——) or 2-mercaptoethanol (- - - -).*
The reaction mixture was prepared the same as Fig. 3. The enzyme was inactivated with 10^{-4} M PCMB for 10 min and afterwards treated with 10 mM DTT (——) or 2-mercaptoethanol (- - - -) for the indicated incubation periods. The arrow shows the addition of thiol resgents.

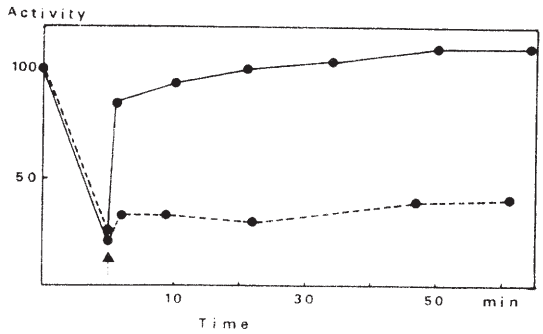

Fig. 5. *Reactivation of DTNB treated PNP by thiol reagents, DTT (——) or 2-mercaptoethanol (- - - -).*
The reaction mixture was prepared the same as Fig. 3. The enzyme was inactivated with 2.5×10^{-4} M DTNB for 10 min and afterwards treated with 10 mM DTT (——) or 2-mercaptoethanol (- - - -) for the indicated incubation periods. The arrow shows the addition of thiol reagents.

of PNP.

Effects of sulfhydryl reagents on kinetic behavior of PNP

It was shown that the low concentration of sulfhydryl reagents inhibited the enzyme only partially and within 30 min DTT completely reactivated the enzyme inhibited by PCMB or DTNB. The kinetic behavior of the residual activity of PCMB-inactivated PNP was examined. As shown in Table 3, the value of K_m was determined to be 1.9×10^{-4} M when inosine was a variable

Table 3. *Comparison of kinetic parameters of PCMB or DTNB nontreated, treated and reactivated PNP when inosine is a variable substrate.*

	K_m of inosine (M)	Relative V_m
PCMB nontreated PNP	1.4×10^{-4}	1.0
PCMB treated PNP	1.9×10^{-4}	0.78
PCMB treated and reactivated PNP	1.4×10^{-4}	1.1
DTNB nontraeted PNP	1.5×10^{-4}	1.0
DTNB treated PNP	2.5×10^{-4}	0.94
DTNB treated and reactivated PNP	1.5×10^{-4}	0.99

substrate.

The value of K_m was higher than that for non-treated PNP and the value of V_m was reverse. When the PCMB inactivated enzyme was reactivated by treatment with DTT, the original kinetic behavior was restored.

A similar pattern was observed for K_m values, but no change was found in V_m values, when the enzyme was inactivated by DTNB and afterwards reactivated by DTT.

Earlier it was shown that substrate activation occurred with PNP in *S. cerevisiae* at high concentration of phosphate (6). This phenomenon could be due to a possible cooperativity among subunits of PNP. When the enzyme was treated with DTNB, no substrate activation was observed with the K_m value of 1.5×10^{-3} M when phos phate was a variable substrate as shown in Fig. 6. The values of K_m of phosphate for the non-treated enzyme were estimated to be 1.1×10^{-3} M and 3.1×10^{-3} M by extrapolation of linear part of Lineweaver-Burk plot. Similar patterns were obtained when the enzyme was treated with PCMB. The K_m of phosphate was determined to be 2.5×10^{-3} M for PCMB-treated enzyme.

Effects of substrates on PCMB or DTNB inactivated PNP

In order to determine whether the substrate have any effects on PCMB or DTNB treated PNP, the enzyme was preincubated for 10 min with each substrate and afterwards treated for 10 min with sulfhydryl reagents. As shown in Table 4, guanosine and deoxyguanosine had no effect on the PCMB-treated PNP. However, ribose 1-phosphate (R-1-P) and phosphate among various substrates showed greatest effect. When the substrates were combined such as R-1-P or phosphate and nucleoside or base, no enhancement of activity was shown.

When the enzyme was inactivated by DTNB after the incubation with each substrate, similar results were obtained. R-1-P and phosphate protected the enzyme from DTNB significantly, whereas guanosine, deoxyguanosine, inosine, deoxyinosine, adenosine, deoxyadenosine and guanine showed no protection.

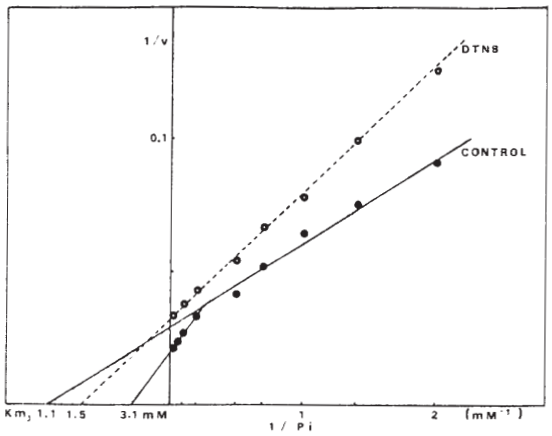

Fig. 6. *Lineweaver-Burk plot of PNP treated or nontreated with DTNB with phosphate as a variable substrate.*
The reaction mixture contained, in a final volume of 1 ml, 50 mM Tris-Cl, pH 7.3, 1 mM of inosine, 0.02 units of xanthine oxidase and the enzyme treated (----) or nontreated (——) with 50 μM DTNB. Phosphate was added in the concentration indicated.

DISCUSSION

Purine nucleoside phosphorylase (PNP) in *S. cerevisiae* has been previously partially characterized (5) and part of kinetic analysis was re ported (6). Further kinetic analysis was done to elucidate the reaction mechanism of PNP. The experiments indicated that the binary complexes of PNP · phosphate and PNP. ribose 1-phosphate were involved in the reaction mechanism. The existence of binary complex of enzyme and phosphate was demonstrated by the competitive inhibition by ribose 1-phosphate when phosphate was a variable substrate. In addition, when inosine was a variable substrate, pentose 1-phophate showed no inhibition at a saturating level of phosphate, but showed a noncompetitive inhibition with low level of phosphate. The initial velocity and product inhibition studies demonstrated above were consistent with the predominant mechanism of the reaction being an ordered bi, bi reaction. The phosphate bound to the enzyme first, followed by nucleoside and base,

Table 4. *Protection of PCMB or DTNB treated PNP by various compounds.*

Preincubation with indicated compounds	Residual PNP activity	
	Inhibited by PCMB	Inhibited by DTNB
Control	13	11
1 mM GR	9	10
1 mM dGR	9	10
1 mM Ino	30	4
1 mM dIno	20	0
0.5 mM Gua	9	5
1 mM Ado	13	11
1 mM dAdo	6	4
2.5 mM R-1-P	52	68
2.5 mM R-1-P+1 mM GR	34	23
2.5 mM R-1-P+1 mM Ado	42	68
2.5 mM R-1-P+1 mM Ino	50	–
50 mM Pi	63	85
50 mM Pi+1 mM Ado	64	75
50 mM Pi+1 mM Gr	36	69
50 mM Pi+1 mM Gua	32	65

The reaction mixture was prepared as described in Materials and Methods.

was the first product to leave, followed by ribose 1-phosphate. When the second substrate, nucleoside, was bound, an isomerization occurred and hypoxanthine was detached afterwards with leaving ribose 1-phosphate as a final product.

In fact, there is a confusing result. When phosphate was a variable substrate at 1 mM of inosine, guanine showed a noncompetitive inhibition pattern. If the level of inosine was saturated, the product would act as an uncompetitive inhibitor. The K_m value of inosine was determined to be 2×10^{-4} M as previously reported for this enzyme. The concentration of inosine at 1 mM appeared not to be saturated enough for the enzyme. Much higher concentration of inosine was not tried because of relatively low solubility of inosine. Substrate binding studies with radioactive nucleoside or phosphate would give more evidence to elucidate the reaction mechanism of PNP in *S. cerevisiae*.

The suggested ordered mechanism was consistent with the mechanism found for PNP from highly purified bovine thyroid (3) and calf spleen PNP (28) with same sequence. However, PNP from calf spleen and human erythrocyte (35) catalyzed ordered bi, bi mechanism with nucleoside binding prior to phosphate. The proposed mechanism for enteric bacteria, *Escherichia coli* and *Salmonella typhimurium* was slightly deviated (13). Nucleoside and phosphate may randomly bind to the enzyme and afterwards products dissociated from the enzyme sequentially, first purine base and then ribose 1-phosphate. Initial velocity rate from bovine brain (18) and human erythrocyte PNP (19) were suggested to be Theorall-Chance mechanism with nucleoside or base bound to the enzyme prior to phosphate or ribose 1-phosphate. The competition between base and nucleoside and between phosphate and ribose 1-phosphate was found. Enzyme from these sources did not obey Michelis Menten kinetics over the whole range of substrate concentrations. This seemed to be due to nonequivalent interacting active sites between subunits (31) or to dissociation of the enzyme by high concentration of substrates (35). Since the order of reaction mechanism was determined from the product inhibition studies, the errors of the sequence for substrate addition were possibly existed with a limited range of substrate concentrations.

Thiol groups in proteins have been widely studied by the process of chemical modification (4, 22, 33). Suitable reagents for this use include N-ethylmaleimide (15), DTNB (32) and haloacetate (23). The kinetically suggested mechanism of PNP in *S. cerevisiae* was in agreement with the results of protection studies against the inactivation of the enzyme by sulfhydryl reagents, PCMB and DTNB. PNP was protected by some of its substrates; ribose 1-phosphate and phosphate, but not by nucleosides or base, supporting the reaction order of ordered bi, bi mechanism. When the phosphate binding site of the enzyme was occupied, the sulfhydryl groups of PNP which PCMB or DTNB could react seemed to be less, resulting in inhibition of the inactivation of the enzyme.

It was demonstrated by Jensen and Nygaard (13) that PNP from *S. typhimurium* was inactivated by PCMB and reversed by excess 2-mercaptoethanol. Inosine and ribose 1-phosphate protected the enzyme, whereas phosphate and adenine had no effect. The protection exerted by ribose 1-phosphate was competed by phosphate, suggesting that three ligands, phosphate, inosine and ribose 1-phosphate could bind to the free enzyme. Their reaction mechanism was more likely to be a random bi, bi with dead end complexes, not an ordered bi, bi. Argawal *et al.* (1) showed that DTNB-inactivated human erythrocytic PNP was protected by hypoxanthine or guanine, not by phosphate or ribose 1-phosphate These results were consistent with the ordered bi, bi mechanism.

Two PNP were purified from vegetative *Bacillus subtilis* cells (12). Two enzymes specific for adenosine and deoxyadenosine were inactivated by PCMB and protected by Pi, adenosine and ribose 1-phosphate.

The sulfhydryl reagents, DTNB or PCMB inactivated purine nucleoside phosphorylase from *S. cerevisiae*. The enzyme was totally reactivated by DTT and partially by 2-mercaptoethanol, indicating that inactivation was reversible. The different effects of DTT and 2-mercaptoethanol appeared to be due to different reactivity to sulfhydryl groups. The kinetic behavior of the PCMB inactivated enzyme had been changed with higher K_m values and lower V_m when inosine was a variable substrate and was completely restored by DTT. Inactivation by DTNB showed similar pattern of K_m value with that by PCMB, but had not changed the V_m value significantly. That also indicated PCMB and DTNB could react different sulfhydryl groups of the enzyme. PNP from bovine spleen was demonstrated to be allosterically regulated (31, 37). Negative cooperativity was shown to inosine and Pi. The activity to inosine was sensitive to reducing thiols and oxidation caused a loss of cooperativity to inosine as well as a 10 fold decreased affinity for inosine. However, oxidation had no effect on either the affinity or cooperativity toward Pi. The reduced cystein appeared to be specific for binding of nucleoside, not for that of Pi in PNP from bovine spleen.

PNP from rabbit brain (17) showed to be su sceptible to inactivation by PCMB and completely restored by treatment with an excess of 2-mercaptoethanol. However, the PCMB-inactivated PNP from bovine brain (18) regained 50% of its original activity on treatment with an excess of 2-mercaptoethanol.

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초 록: *Saccharomyces cerevisiae*에서 얻은 Purine Nucleoside Phosphorylase의 반응기작과 효소에 대한 Sulfhydryl Reagent의 영향

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*Saccharomyces cerevisiae*에서 얻은 purine nucleoside phosphorylase (PNP)의 반응 기작을 밝히기 위해 반응속도론적 분석이 수행되어졌다. 반응기작에 PNP·phosphate와 PNP·ribose 1-phosphate의 binary complex가 형성되는 것으로 추정되어진다. Initial velocity와 product inhibition study의 결과는 반응이 ordered bi, bi reaction으로 일어난다는 것과 일치하고 있다. 두개의 기질중 무기인산이 효소에 먼저 붙고, 그 다음에 nucleoside, 그리고 base가 효소를 떠나는 첫번째 생성물이고 마지막으로 ribose 1-phosphate가 생성되고 효소는 원래의 상태로 돌아간다. 반응속도론적 분석에 의해 제안된 작용기작은 sulfhydryl reagents인 p-chloromercuribenzoate (PCMB) and 5,5′-dithiobisnitrobenzoate (DTNB)에 의한 효소의 불활성화에 대한 기질의 보호작용의 결과와 일치하고 있다. PNP는 ribose 1-phosphate와 phosphate에 의해 보호되지만 nucleoside나 base에 의해서는 아무런 효과가 없다는 사실은 반응 순서가 효소에 무기인산이 먼저 붙는 ordered bi, bi 기작이라는 것을 지지하고 있다. PCMB 나 DTNB에 의해 불활성화된 PNP는 dithiothreitol (DTT)에 의해서는 활성이 완전히 회복되고 2-mercaptoethanol에 의해서는 77%의 활성이 회복된다는 사실은 효소의 불활성화가 가역적이라는 것을 시사하고 있다. PCMB에 의해 불활성화된 효소는 inosine이 변화하는 기질일때 정상효소보다 높은 K_m과 낮은 V_m 값을 보여주고 이런 현상은 DTT 처리시 원래의 상태로 돌아온다. DTNB에 의해 불활성화된 효소는 PCMB 처리시와 비슷하게 정상효소보다 높은 K_m 값을 보이지만 V_m 값은 큰 변화가 없다. *S. cerevisiae* PNP에서 발견되는 높은 무기인산의 농도에서의 하위단위체간의 음성적 협동성이 PCMB나 DTNB를 처리한 PNP에서는 보이지 않았다.