Rabbit Erythrocyte Purine Nucleoside Phosphorylase

INITIAL-VELOCITY STUDIES

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1. Concave-downward double-reciprocal plots were obtained for rabbit erythrocyte purine nucleoside phosphorylase when the concentration of P1 was varied over a wide range at a fixed saturating concentration of either inosine or deoxyinosine. Similar behaviour was also displayed by the calf spleen enzyme. 2. The degree of curvature of double-reciprocal plots was greatly modified by the presence of SO4\(^{2-}\), introduced into the assay mixture with the linking enzyme xanthine oxidase; competitive inhibition by SO4\(^{2-}\) was observed over a narrow range of high P1 concentrations. 3. Partial inactivation with 5,5'-dithiobis-(2-nitrobenzoic acid) resulted in a marked alteration in the kinetic properties of the enzyme when P1 was the variable substrate. 4. Initial-velocity data are expressed in the form of Hill plots, and the significance of such plots is discussed.

Purine nucleoside phosphorylase (purine nucleoside-orthophosphate ribosyltransferase, EC 2.4.2.1) from various sources has been shown to display nonlinear double-reciprocal plots when the concentration of inosine is varied over a wide range at a fixed saturating concentration of the second substrate, P1 (Kim et al., 1968; Turner et al., 1971; Agarwal et al., 1975; Savage & Spencer, 1977). For the human enzyme, the effect of varying the concentration of phosphate at a fixed concentration of inosine has only been investigated over a very narrow range of high P1 concentrations (Kim et al., 1968), and double-reciprocal plots appeared to be linear within this range.

In most of the studies on purine nucleoside phosphorylase previously reported, xanthine oxidase is utilized in the enzyme-linked assay method devised by Kalckar (1947). It seems inevitable that (NH4)\(_2\)SO4 was introduced into the assay mixture in these studies, since commercial preparations of xanthine oxidase are usually supplied as suspensions in 65%-%-satd. (NH4)\(_2\)SO4 solution. Possible effects of (NH4)\(_2\)SO4 on the initial velocity of the reaction catalysed by purine nucleoside phosphorylase do not appear to have been considered.

In the present study, using purine nucleoside phosphorylase from rabbit erythrocytes and calf spleen, the P1 concentration range has been considerably extended in order to reveal any deviation from linearity that might occur at low P1 concentrations. Furthermore, the inhibition of purine nucleoside phosphorylase by (NH4)\(_2\)SO4 is reported and the effect of using SO4\(^{2-}\)-free xanthine oxidase on the shape of double-reciprocal plots, with P1 as the variable substrate, is considered. An analysis of initial-velocity data in the form of Hill plots is presented and the usefulness of such plots is discussed.

Experimental

Chemicals

Inosine and 2'-deoxyinosine were obtained from BDH, Poole, Dorset, U.K.

Purine nucleoside phosphorylase

Purine nucleoside phosphorylase was partially purified from rabbit erythrocytes to a specific activity of 25 units/mg by using a procedure similar to that previously described (Savage & Spencer, 1977), except that DEAE-Sephadex was replaced by DEAE-Sephadex. The enzyme was stored in 50mM-Tris/HCl buffer, pH7.5, at 4°C, and used within 3 days of the final purification step. No detectable loss of activity occurred during this time. Calf spleen purine nucleoside phosphorylase was supplied by Boehringer, Mannheim, Germany.

Treatment of xanthine oxidase

Xanthine oxidase (Boehringer) supplied as a suspension in 65%-%-satd. (NH4)\(_2\)SO4 solution, was collected by centrifugation at 6000rev./min for 30 min by using an MSE 18 centrifuge and dissolved in 50mm-Tris/HCl buffer, pH 7.5. The solution was extensively dialysed against several changes of the same buffer and finally concentrated to a small volume by using an Amicon Diaflo ultrafiltration cell (Amicon, High Wycombe, Bucks., U.K.) fitted with a UM 10 filter. Where untreated xanthine oxidase was used, the enzyme was used directly as supplied.

Purine nucleoside phosphorylase activity

The assay of enzymic activity and definition of the enzyme unit used was as previously described (Savage & Spencer, 1977).
Results

Initial-rate plots with $P_i$ as the variable substrate

Previous kinetic studies on human erythrocyte purine nucleoside phosphorylase with $P_i$ as the variable substrate at a fixed saturating concentration of inosine gave apparently linear double-reciprocal plots over the narrow range of high concentrations examined (Kim et al., 1968). At very low $P_i$ concentrations (approx. 0.02 mM), the presence of phosphate ion impurities in the assay mixture may contribute significantly to the overall observed reaction rate, and so care must be taken to avoid $P_i$ contamination in studies of this type when the $P_i$ concentration range is extended to include much lower values than those previously studied for the human erythrocyte enzyme.

A major potential source of $P_i$ contamination is the linking enzyme xanthine oxidase; indeed we found that some commercial preparations of this enzyme gave appreciable 'residual' reaction rates in the complete absence of added $P_i$. It is therefore important to ensure that all enzyme preparations and buffer solutions are essentially free from phosphate ion contamination, especially when initial-velocity measurements are made at low $P_i$ concentrations. For this reason, extensively dialysed xanthine oxidase was used in the present study.

When the concentration of $P_i$ was varied over a wide range at a fixed saturating concentration of either inosine or 2'-deoxyinosine, non-linear double-reciprocal plots were obtained (Fig. 1). In these experiments, extensively dialysed xanthine oxidase was present in an amount in excess of that required for the maximum rate of conversion of the product hypoxanthine into uric acid; addition of further xanthine oxidase did not affect the results shown in Fig. 1 at either high or low concentrations of $P_i$. Furthermore, zero reaction rates were observed in the absence of added $P_i$, indicating that $P_i$ contamination of the assay mixture did not occur under the conditions used.

Inhibition by $SO_4^{2-}$

When untreated xanthine oxidase was used in the assay mixture under conditions identical with those described for Fig. 1, the degree of curvature of double-reciprocal plots was greatly modified; although initial velocities measured at high $P_i$ concentrations were unaffected, measurements made at low $P_i$ concentrations were considerably lower than those obtained under similar conditions when extensively dialysed xanthine oxidase was used. Consequently, double-reciprocal plots appeared to be approximately linear over the entire range of $P_i$ concentrations shown in Fig. 1. The presence of an inhibitor of purine nucleoside phosphorylase in the untreated xanthine oxidase could account for this observation, and, since the enzyme is supplied as a suspension in 65% satd. 

(NH$_4$)$_2$SO$_4$ solution, it seemed a likely possibility that inhibition by $SO_4^{2-}$ with respect to $P_i$ may occur when untreated xanthine oxidase is used. To test this possibility, the effect of (NH$_4$)$_2$SO$_4$ on the initial velocity with $P_i$ as the variable substrate was investigated; competitive inhibition patterns were observed over the limited, high $P_i$ concentration range studied (Fig. 2). Identical results were obtained when Na$_2$SO$_4$ was substituted for (NH$_4$)$_2$SO$_4$, indicating that SO$_4^{2-}$ is the inhibiting species, since Na$^+$ ions are a normal component of the reaction mixture.

Previous kinetic studies on rabbit erythrocyte purine nucleoside phosphorylase showed that non-linear double-reciprocal plots were obtained when the concentration of inosine was varied over a wide range at a fixed saturating concentration of $P_i$ (Savage & Spencer, 1977). When these experiments were repeated with extensively dialysed xanthine oxidase, results were obtained identical with those previously reported when untreated xanthine oxidase was used. This result would be expected, since the fixed concentration of $P_i$ used in these experiments (50 mM) was sufficiently high to prevent any observable inhibition by SO$_4^{2-}$, present only at a comparatively low concentration (approx. 10 mM).

Failure to observe non-linearity in double-reciprocal plots for human purine nucleoside phosphorylase...
with $P_1$ as the variable substrate may have been due to the very narrow range of $P_1$ concentrations investigated (Kim et al., 1968). A further factor that may influence the shape of such plots is the presence of $SO_4^{2-}$, introduced into the assay mixture with xanthine oxidase. A range of $P_1$ concentrations similar to those shown in Fig. 2 was used by Kim et al. (1968) in studies on the reaction mechanism of human purine nucleoside phosphorylase. Clearly it is necessary to exclude $SO_4^{2-}$ from the reaction mixture for the determination of kinetic constants when $P_1$ is the variable substrate. Furthermore, the effect of $SO_4^{2-}$ inhibition will be more pronounced at lower $P_1$ concentrations and may thus 'mask' any non-linearity that would otherwise be observed when the $P_1$ concentration range is extended to lower values.

**Studies with calf spleen purine nucleoside phosphorylase**

Calf spleen purine nucleoside phosphorylase was supplied as a suspension in 60% $\text{satd.} (NH_4)_2SO_4$ solution and therefore was extensively dialysed to remove $SO_4^{2-}$ and the enzyme stored in 50mM-Tris/HCl buffer, pH 7.5, before use.

It has been shown that bovine erythrocyte purine nucleoside phosphorylase displays completely linear double-reciprocal plots with both inosine and 2'-deoxyinosine as the variable substrate in the presence of a saturating concentration of $P_1$ (Agarwal et al., 1975). However, bovine spleen purine nucleoside phosphorylase

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**Fig. 2. Inhibition of rabbit erythrocyte purine nucleoside phosphorylase by $(NH_4)_2SO_4$**

The reaction mixture contained 50mM-Tris/HCl buffer, pH 7.5, 0.06 EC unit of dialysed xanthine oxidase, 2$\mu$l of rabbit erythrocyte purine nucleoside phosphorylase (concentration 27$\mu$g/ml and specific activity 25 units/mg) and various amounts of NaH$_2$PO$_4$ and (NH$_4$)$_2$SO$_4$ in a final volume of 3.5 ml at 30°C. The concentration of (NH$_4$)$_2$SO$_4$ is indicated alongside the appropriate line.

**Fig. 3. Effect of concentration of inosine or 2'-deoxyinosine on the initial velocity of the reaction catalysed by calf spleen and rabbit erythrocyte purine nucleoside phosphorylase**

The reaction mixture contained 50mM-sodium phosphate buffer, pH 7.5, 0.06 EC unit of dialysed xanthine oxidase, various amounts of inosine (●) or 2'-deoxyinosine (○) in a final volume of 3 ml at 30°C, and either (a) 2$\mu$l of dialysed calf spleen purine nucleoside phosphorylase (concentration 180$\mu$g/ml of 50mM-Tris/HCl buffer, pH 7.5) or (b) 5$\mu$l of rabbit erythrocyte purine nucleoside phosphorylase (concentration 150$\mu$g/ml and specific activity 25 units/mg).
phosphorylase displayed linear plots with inosine, but concave-downward plots with 2'-deoxyinosine as the variable substrate (Agarwal & Parks, 1969; Agarwal et al., 1975). Calf spleen purine nucleoside phosphorylase was found to display kinetic behaviour similar to that reported by Agarwal et al. (1975) for the bovine spleen enzyme; non-linear double-reciprocal plots were only observed with 2'-deoxyinosine as the variable substrate (Fig. 3). In contrast, non-linear plots were obtained with both inosine and 2'-deoxyinosine when rabbit erythrocyte purine nucleoside phosphorylase was used under similar conditions (Fig. 3). Furthermore, non-linear plots were obtained for calf spleen purine nucleoside phosphorylase when P_i was the variable substrate, irrespective of whether the fixed, saturating substrate was inosine or 2'-deoxyinosine (Fig. 4), provided that the concentration of P_i was varied over a wide range of values and the assay mixture was essentially free from SO_4^{2-} contamination.

**Effect of 5,5'-dithiobis-(2-nitrobenzoic acid)**

Previous studies have shown that both human and rabbit purine nucleoside phosphorylase are partially inactivated in a time-dependent manner on incubation with excess 5,5'-dithiobis-(2-nitrobenzoic acid) (Agarwal & Parks, 1971; Savage & Spencer, 1977). Kinetic studies with the partially inactivated enzyme revealed completely linear double-reciprocal plots with inosine as the variable substrate. In the present study, partially purified rabbit erythrocyte purine nucleoside phosphorylase was treated with 5,5'-dithiobis-(2-nitrobenzoic acid) in the manner previously described (Savage & Spencer, 1977) and the partially inactivated enzyme extensively dialysed against 50mM-Tris/HCl buffer, pH 7.5. The treated enzyme was then used to investigate the effect of varying the P_i concentration on initial reaction rates. In contrast with the previous studies with inosine as the variable substrate, non-linearity was not completely abolished (Fig. 5). Control values were obtained by using untreated purine nucleoside phosphorylase, diluted to give the same reaction rate as the treated enzyme at saturating phosphate concentrations.

**Further analysis of initial-velocity data**

Information about the details of the binding process can often be inferred from Hill plots, which utilize initial-velocity data. When used in this manner, it is assumed that the steady-state velocity is proportional to the fractional saturation of the enzyme at equilibrium, and the Hill equation (Hill, 1910) takes the following form (Monod et al., 1963);

\[
\log \left( \frac{v}{V_{\text{max}} - v} \right) = h \log [S] + \log K
\]

where \( v \) and \( V_{\text{max}} \) are initial and maximum velocities respectively, [S] is the substrate concentration and \( K \) and \( h \) are constants. When \( \log [v/(V_{\text{max}} - v)] \) is plotted against \( \log [S] \), the slope of the Hill plot (the Hill coefficient, \( h \)) varies according to the mechanism of the binding process. The Hill coefficient may be considered as a parameter that measures the extent of interaction between multiple interdependent sites (Wyman, 1967); values of \( h \) greater than unity indicate positive co-operativity, whereas values less than unity

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**Fig. 4. Effect of concentration of P_i on the initial velocity of the reaction catalysed by calf spleen purine nucleoside phosphorylase**

Conditions were as described for Fig. 1, except that 2μg of dialysed calf spleen purine nucleoside phosphorylase was used (concentration 200μg/ml of 50mm-Tris/HCl buffer, pH 7.5). ●, Initial velocity at a fixed concentration (2.0mm) of inosine; ○, Initial velocity at a fixed concentration (2.0mm) of 2'-deoxyinosine.
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Fig. 5. Double-reciprocal plots, with $P_1$ as the variable substrate for rabbit erythrocyte purine nucleoside phosphorylase, after partial inactivation with 5,5'-dithiobis-(2-nitrobenzoic acid)

Conditions for the partial inactivation of rabbit erythrocyte purine nucleoside phosphorylase with 5,5'-dithiobis-(2-nitrobenzoic acid) were as previously described (Savage & Spencer, 1977). The conditions for the assay of purine nucleoside phosphorylase activity were as described for Fig. 1. $\square$, Initial velocity of partially inactivated purine nucleoside phosphorylase; $\bullet$, initial velocity of untreated purine nucleoside phosphorylase (control).

Data are expressed (i.e. in the form of the double-reciprocal plot); the assumption that linearity in such plots represents a region where classical Michaelis-Menten kinetics predominate is clearly unfounded. Further, the mechanism responsible for the observed non-linearity is not restricted to regions of high substrate concentrations, implied by the phrase 'substrate activation at high substrate concentrations' (Agarwal & Parks, 1969), which was used to describe the non-linear plots observed with the human enzyme with inosine as the variable substrate; as shown in Fig. 6 it can be demonstrated over a much wider range of substrate concentrations.

When the initial-velocity data were expressed in the form of Hill plots, almost identical plots were obtained, irrespective of whether the fixed substrate was inosine (Fig. 7) or 2'-deoxyinosine; for both fixed substrates the slope at intermediate $P_1$ concentrations was about 0.7.

In the analysis of ligand-binding data, it has been shown that the asymptotes of Hill plots at the ex-

![Graphical representation]

Fig. 6. Double-reciprocal plots with $P_1$ as the variable substrate using rabbit erythrocyte purine nucleoside phosphorylase

Conditions were as described for Fig. 1. The results are expressed over two principal ranges: (a) 0.1–50.0mM-phosphate; (b) 0.005–50.0mM-phosphate.

$\bullet$, Initial velocity at a fixed concentration (2.0mM) of inosine; $\bigcirc$, Initial velocity at a fixed concentration (2.0mM) of 2'-deoxyinosine.
Discussion

In the present study, non-linear double-reciprocal plots with P₁ as the variable substrate were only observed when initial-velocity data were obtained over a wide range of P₁ concentrations, and then only if the assay mixture was essentially free from SO₄²⁻ contamination. Failure to meet these conditions may result in apparently linear double-reciprocal plots, similar to those reported for human purine nucleoside phosphorylase with P₁ as the variable substrate (Kim et al., 1968).

It was proposed previously that the non-linear double-reciprocal plots obtained for rabbit erythrocyte purine nucleoside phosphorylase with inosine as the variable substrate may reflect negative co-operative binding of the substrate (Savage & Spencer, 1977). Although double-reciprocal plots that are concave-downward are often characteristic of enzymes that display negative co-operative binding of substrate, diagnostic evaluation in favour of such a mechanism can only be justified when other possible explanations are reasonably excluded. No evidence was found for the occurrence of multiple molecular forms of rabbit erythrocyte purine nucleoside phosphorylase, and furthermore, the enzyme showed no tendency to dissociate in the presence of saturating concentrations of either or both substrates, indicating that dissociation of the enzyme into active subunits, with different kinetic properties, does not occur (Savage & Spencer, 1977). It is concluded, then, that the shape of the double-reciprocal plots reflects a real kinetic property of the rabbit enzyme in the phosphorolysis of inosine and 2'-deoxyinosine under the conditions used in these experiments and cannot adequately be ascribed to the presence of more than one active molecular form of the enzyme.

As a consequence of the non-linear double-reciprocal plots observed with rabbit erythrocyte purine nucleoside phosphorylase, reaction-sequence and product-inhibition studies must be confined to regions representing very narrow ranges of variable substrate concentrations where double-reciprocal plots appear to be linear. The limitations inherent in enzyme studies of this type should be recognized; the predominant reaction mechanism inferred from studies performed over a narrow range of substrate concentrations may not necessarily represent that which predominates when the substrate concentration is varied over a much wider range of values. With this in mind, an ordered sequential reaction sequence was inferred for the addition of substrates to rabbit erythrocyte purine nucleoside phosphorylase under the limiting conditions used (Savage, 1978), and product-inhibition studies were consistent with a mechanism where the nucleoside is the first substrate to add to the enzyme and the purine base the last product to leave the enzyme surface. Further, the inhibition patterns obtained were consistent with a Theorell–Chance mechanism (Theorell & Chance, 1951; Cleland, 1963, 1970) where the formation of intermediate complexes either does not occur or is not rate-limiting. Kinetic studies on human erythrocyte...
purine nucleoside phosphorylase have also indicated that the phosphorolytic reaction proceeds via an ordered, sequential reaction sequence, and moreover, binding studies showed that guanosine, but not orthophosphate, readily binds to the native enzyme (Kim et al., 1968). Also, in the following paper (Savage & Spencer, 1979) and elsewhere (Savage, 1978), evidence is presented that indicates that inosine and guanosine readily bind to the native rabbit enzyme, whereas orthophosphate under similar conditions will apparently only bind to the enzyme that already has the nucleoside bound.

It may be pertinent at this point to mention that a random reaction sequence may give rise to non-linear double-reciprocal plots (Dalziel, 1958), since such a mechanism provides the possibility of more than one enzymic form with which the substrate may react on the way to products. However, in view of the above findings, it seems unlikely that a random reaction sequence contributes in a kinetically significant way to the observed non-linear plots. It therefore seems reasonable to propose that the kinetic behaviour displayed by rabbit erythrocyte purine nucleoside phosphorylase reflects negative, antagonistic, homotropic interactions between the active sites of the enzyme; the results are consistent with a model similar to that proposed by Conway & Kosland (1968) in which the binding of substrate to one subunit induces changes in the other subunits resulting in a decrease in affinity between the substrate and vacant sites of the neighbouring subunits of the enzyme. The symmetrical model of Monod et al. (1965) apparently cannot explain such kinetic behaviour.

The expression of substrate-binding data in the form of a Hill plot can often yield information about the details of the binding process. In the present study, the fractional saturation (that is the fraction of the total number of binding sites occupied by the substrate) is estimated as $v/V_{\text{max}}$, where $v$ and $V_{\text{max}}$ are the initial and maximum velocities respectively. Although initial-velocity data are frequently manipulated in this way, it must be remembered that it is necessarily assumed that $v$ is proportional to the fractional saturation over the entire range of substrate concentrations studied, an assumption that clearly warrants verification before assessment of such plots can be reasonably justified. However, there are many cases in which the assumption has been correct (Cornish-Bowden & Koshland, 1975), indicating that expression of initial-velocity data in the form of Hill plots can often provide a valuable basis for further binding studies.

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