Rabbit Erythrocyte Purine Nucleoside Phosphorylase

DIFFERENTIAL-INACTIVATION STUDIES

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1. Qualitative studies on the stability of rabbit erythrocyte purine nucleoside phosphorylase showed a marked decrease in the susceptibility of the enzyme to thermal inactivation and digestion by proteinases of different specificities in response to certain of its substrates. 2. The extent to which inosine stabilizes the enzyme against thermal and proteolytic inactivation is related in a quantitative manner to the concentration of this substrate; it is proposed that differences in the rates of inactivation of the enzyme may reflect substrate-induced conformational changes in the enzyme structure that could alter the binding properties of the enzyme in a kinetically significant way. 3. A synergistic effect in the stabilization of the enzyme is observed in response to both substrates, inosine and phosphate, when the enzyme is inactivated with Pronase. 4. In the presence of substrate an increased rate of inactivation after reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) is reported. 5. Differential-inactivation studies were also carried out with calf spleen purine nucleoside phosphorylase, and the results are discussed in relation to the kinetic properties displayed by this enzyme.

The binding of substrates or substrate analogues to an enzyme is often accompanied by a readily detectable change in its susceptibility to proteinase digestion and thermal inactivation (Zyk *et al.*, 1969; Adelman *et al.*, 1968; Jacobs & Cunningham, 1968; Rupley, 1967; Lui & Cunningham, 1966; Trayser & Colowick, 1961). It has been proposed by Zyk *et al.* (1969) that the binding of such ligands may cause a change in the conformation of the enzyme structure that is reflected in stabilization towards proteolysis and thermal inactivation, and the term 'conformative response' has been suggested by Citri & Zyk (1967) to describe conformational changes that accompany specific ligand binding. In the preceding paper (Savage & Spencer, 1979) and elsewhere (Savage & Spencer, 1977) it was proposed that negative homotropic interactions may occur between the active sites of rabbit erythrocyte purine nucleoside phosphorylase when the concentration of either substrate is varied at a fixed saturating concentration of the second substrate. The kinetic data reported in both studies (Savage & Spencer, 1977, 1979) are consistent with a model in which the binding of each substrate molecule causes conformational changes in the enzyme structure that lead to a decrease in affinity between the substrate and vacant sites on neighbouring subunits.

In the present paper we describe the results of differential-inactivation studies on rabbit erythrocyte purine nucleoside phosphorylase. It was thought that studies of this type might provide an insight into the mechanism of the apparent negative co-operative

effect observed during kinetic studies. Furthermore, the elucidation of substrate-induced conformational changes in rabbit erythrocyte purine nucleoside phosphorylase would provide evidence in support of the sequential mechanism proposed by Conway & Koshland (1968) for the negative co-operative binding of substrate. The effects of substrates on the susceptibility of calf spleen purine nucleoside phosphorylase to thermal inactivation and proteolysis were also investigated; a consistent relation between the behaviour observed during differential-inactivation studies and that observed during kinetic studies is reported.

Experimental

Chemicals

Inosine was supplied by BDH, Poole, Dorset, U.K. Guanosine, xanthosine, adenosine, 2'-deoxyinosine, hypoxanthine and guanine were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Enzymes

Trypsin (B grade) was obtained from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.) and Pronase (B grade) was from Calbiochem (La Jolla, CA, U.S.A.). Rabbit erythrocyte purine nucleoside phosphorylase (specific activity 22–26 EC units/mg) was partially purified in the manner described in the preceding paper (Savage & Spencer, 1979) and stored

at 4°C in 50mM-Tris/HCl buffer, pH7.5. Calf spleen purine nucleoside phosphorylase and xanthine oxidase was supplied by Boehringer (Mannheim, Germany).

Purine nucleoside phosphorylase activity

This was measured by using the xanthine oxidase-linked method previously described (Savage & Spencer, 1977), except that xanthine oxidase was extensively dialysed against 50mM-Tris/HCl buffer, pH7.5, before use in the assay mixture.

Pronase treatment

Pronase solution, (1.0mg/ml of 10mM-CaCl₂) was freshly prepared before each experiment and kept at 4°C. Partially purified rabbit erythrocyte purine nucleoside phosphorylase in 50mM-Tris/HCl buffer, pH7.5 (0.1ml), was diluted with the same buffer (0.2ml) containing an appropriate amount of substrate or substrate analogue to give the required concentration in a final volume of 0.3ml. The solution was incubated at 37°C, Pronase solution (5–10 µl) was added and the solution thoroughly mixed. Samples (2 µl) were removed from the incubation mixture after various time intervals and immediately assayed for purine nucleoside phosphorylase activity. Control samples, without Pronase, were treated in an identical manner to check for any inactivation caused by the incubation conditions used.

Trypsin treatment

Trypsin solution (10mg/ml of 25mM-CaCl₂) was freshly prepared before each experiment and kept at 4°C. The procedure was identical with that described for Pronase above.

Heat treatment

Partially purified rabbit erythrocyte purine nucleoside phosphorylase in 50mM-Tris/HCl buffer, pH7.5 (0.1 ml), was diluted with the same buffer containing an appropriate amount of substrate to give the desired concentration in a final volume of 0.3ml. Solutions were incubated at 63.5°C and samples (2 µl) removed after various time intervals and immediately assayed for purine nucleoside phosphorylase activity.

Results

Qualitative studies

No inactivation was observed when the enzyme was incubated with either Pronase or trypsin at room temperature (19°C). An incubation temperature of 37°C was necessary to obtain an appreciable rate of

inactivation. Controls, with Pronase or trypsin absent, and at various concentrations of substrate, did not show any marked decrease in activity when incubated at 37°C over the time period studied. Hence any observed inactivation of the enzyme at this temperature was due to proteolysis and not due, even in part, to thermal inactivation.

The effect of inosine and P_i on the rate of inactivation of rabbit purine nucleoside phosphorylase is shown in Fig. 1. Inactivation by Pronase was greatly decreased in the presence of an excess of either substrate, and almost complete protection was obtained when saturating concentrations of both substrates were present in the incubation mixture. Similar results were obtained by using trypsin in place of Pronase and by thermal inactivation at 63.5°C.

Quantitative studies

It has been shown by Zyk *et al.* (1969) that if the conformational response to the binding of a ligand alters the stability of the enzyme, then the fraction of the enzyme that has responded (F_{cr}) can be expressed as follows:

$$F_{cr} = \frac{E'}{E_t} = \frac{E_t - E}{E_t} = 1 - \frac{E}{E_t} \quad (1)$$

where E' and E are the concentrations of the enzyme in the altered and native conformations respectively and E_t is the total concentration of the enzyme. Further-

Fig. 1. Effect of saturating concentrations of inosine and P_i on the rate of inactivation of rabbit erythrocyte purine nucleoside phosphorylase by Pronase

The enzyme (17 µg of protein in 50mM-Tris/HCl buffer, pH7.5, specific activity 26 units/mg), in a final volume of 0.3ml, was incubated with Pronase (5 µg) and saturating concentrations of the substrates indicated below. Experimental details are described in the text. ○, 2.0mM-Inosine; □, 50.0mM-NaH₂PO₄; ■, 2.0mM-inosine + 50.0mM-NaH₂PO₄; ●, no substrates.

more, the following expression has been derived that utilizes data obtained from differential-inactivation-rate measurements (Zyk *et al.*, 1969):

$$F_{cr} = \frac{1 - (V/V_0)}{1 - (V_s/V_0)} \quad (2)$$

where V_s and V_0 are the observed rates of inactivation of the enzyme in the presence of a saturating amount of ligand or in the complete absence of ligand respectively, and V is the observed rate of inactivation of the enzyme in the presence of a given non-saturating amount of ligand. Thus the fraction of the enzyme that has responded to a given concentration of ligand can be determined at various ligand concentrations by measuring the parameters V , V_0 and V_s .

Fig. 2 shows the variation in the rate of inactivation of rabbit purine nucleoside phosphorylase with the concentration of inosine by using three different methods of inactivation. The rates of inactivation in the absence of inosine (V_0) or in the presence of a saturating concentration of inosine (V_s) were calculated from the linear slopes obtained by plotting the logarithm of residual enzymic activity against incubation time. Similarly, the observed rate of inactivation in the presence of 10 μ M-inosine was calculated from the corresponding slopes shown in Fig. 2. The F_{cr} values corresponding to 10 μ M-inosine were calculated for each of the three criteria used and the results summarized in Table 1.

Table 1 shows that the fraction of the enzyme that has responded to the presence of 10 μ M-inosine under the conditions used is approx. 50%; the values obtained for F_{cr} by the three independent criteria used are in fairly close agreement. It appears, therefore, that the proportion of the enzyme that is stabilized by inosine is a function of the concentration of this substrate and is independent of the criteria used for testing the stability. Hence it seems reasonable to propose that a change in the rate of inactivation by any of the three treatments reflects a substrate-induced change in the conformation of the enzyme. Almost identical F_{cr} values for 10 μ M-inosine were obtained by using the trypsin and Pronase treatments, indicating that the rate of inactivation is retarded to the same extent by the presence of 10 μ M-inosine and is apparently independent of the specificity of the proteinase used. This renders unlikely any effects caused by the 'masking'

Fig. 2. Effect of concentration of inosine on the rate of inactivation of rabbit erythrocyte purine nucleoside phosphorylase

The enzyme (15 μ g of protein in 50 mM-Tris/HCl buffer, pH 7.5, specific activity 25 units/mg), in a final volume of 0.3 ml, was incubated with various concentrations of inosine as indicated. (a) Inactivation by Pronase (5 μ g). (b) Inactivation by trypsin (50 μ g). (c) Inactivation by heat. Experimental details are described in the text.

Table 1. Effect of concentration of inosine on the rate of inactivation of rabbit erythrocyte purine nucleoside phosphorylase
For details see the text.

Treatment	V (for 10 μ M-inosine)	V_s	V_0	V/V_0	V_s/V_0	F_{cr} (for 10 μ M-inosine)
Heat	0.0111	0.00670	0.0150	0.7460	0.449	0.4570
Pronase	0.0223	0.01230	0.0366	0.6082	0.336	0.5898
Trypsin	0.0061	0.00296	0.0105	0.5844	0.283	0.5800

of a critical bond by inosine at the active site of the enzyme.

Effect of alternative substrates and substrate analogues

A greater conformational response was afforded by guanosine compared with inosine under identical conditions when the rabbit enzyme was inactivated by Pronase (Fig. 3). Although an appreciable difference in the maximum response was observed between guanosine and inosine when these substrates were present at saturating concentrations, the F_{cr} value calculated for $10\mu\text{M}$ -guanosine was 0.52, which is similar to the F_{cr} value of 0.58, obtained under similar conditions, for $10\mu\text{M}$ -inosine. Thus it appears that at concentrations of $10\mu\text{M}$ of either nucleoside, approx. 50–60% of the enzyme will be in an altered conformation under the conditions used, and therefore any differences in the susceptibility to inactivation may result from a difference in the magnitude of conformational changes rather than from differences in the degree of saturation of the enzyme.

Marked differences in the maximum conformational response were also observed when adenosine and xanthosine were used (Fig. 3). All nucleosides were at

Fig. 3. Effect of hypoxanthine and various purine nucleosides on the rate of inactivation of rabbit erythrocyte purine nucleoside phosphorylase by Pronase

The enzyme ($15\mu\text{g}$ of protein in 50mM -Tris/HCl buffer, pH 7.5, specific activity 25 units/mg), in a final volume of 0.3ml , was incubated with Pronase ($10\mu\text{g}$) and a saturating concentration (2.0mM) of a given purine nucleoside, or hypoxanthine, or in the absence of substrates (control) as indicated. Experimental details are described in the text.

saturating concentrations; increasing the concentration did not affect the conformational response displayed by a given nucleoside. A more dramatic difference was observed when hypoxanthine was used in the incubation mixture. In this case, hypoxanthine is the purine-base moiety present in inosine, and yet a much greater conformational response is afforded by hypoxanthine compared with inosine under identical conditions. Since both these substrates were present at saturating concentrations in these experiments, it seems likely that the observed effect is due to an increase in the magnitude of conformational changes induced by hypoxanthine compared with inosine. The possibility that the substrate masks a critical bond at the active site is also rendered unlikely by these observations.

Synergistic effect of two substrates

When the native enzyme was incubated with various concentrations of NaH_2PO_4 , no appreciable protection against inactivation by Pronase was observed below a concentration of 2.0mM (Fig. 4). However, in the presence of a saturating concentration of inosine, P_i concentrations as low as $10\mu\text{M}$ resulted in a marked increase in stability towards inactivation by Pronase under identical conditions. These findings suggest that

Fig. 4. Synergistic effect of both substrates on the rate of inactivation of rabbit erythrocyte purine nucleoside phosphorylase by Pronase

The enzyme ($15\mu\text{g}$ of protein in 50mM -Tris/HCl buffer, pH 7.5, specific activity 23 units/mg), in a final volume of 0.3ml , was incubated with Pronase ($5\mu\text{g}$) and various amounts of inosine and NaH_2PO_4 or in the absence of substrates (control) as indicated. Experimental details are described in the text.

conformational changes may accompany the binding of P_i to the enzyme when in the presence of a saturating concentration of inosine; implicit in these observations is the fact that P_i apparently binds more readily to the enzyme that already has inosine bound than it does to the native enzyme.

Differential inactivation by 5,5'-dithiobis-(2-nitrobenzoic acid)

When partially purified rabbit erythrocyte purine nucleoside phosphorylase in sodium phosphate buffer was incubated with excess 5,5'-dithiobis-(2-nitrobenzoic acid), partial inactivation occurred and a residual activity, representing approx. 30% of the initial activity, remained after about 60min (Savage & Spencer, 1977). When similar experiments were performed with the enzyme in 50mM-Tris/HCl buffer, pH 7.5, instead of phosphate buffer, a biphasic pattern of inactivation was observed (Fig. 5). Complete

activity was restored after 40min by treatment with excess dithiothreitol. The presence of excess inosine or hypoxanthine in the reaction mixture had little effect on the first phase of inactivation under these conditions, but protection against inactivation was observed during the second phase.

By using lower concentrations of 5,5'-dithiobis-(2-nitrobenzoic acid), the rate of inactivation during the initial phase could be slowed down to such an extent that a more detailed investigation of this region was possible. Under these conditions an increase in the rate of inactivation was observed during the initial phase when the enzyme was incubated with excess inosine or hypoxanthine, followed by a decrease in rate during the second phase of inactivation (Fig. 5).

Differential inactivation of calf spleen purine nucleoside phosphorylase

Differential-inactivation studies on calf spleen purine nucleoside phosphorylase showed that a greater protection against inactivation by Pronase is afforded by 2'-deoxyinosine compared with inosine under identical conditions (Fig. 6). Similar results were also obtained when the calf spleen enzyme was subjected to thermal inactivation. In contrast with these findings, very little difference in the rate of inactivation of rabbit erythrocyte purine nucleoside phosphorylase was observed when saturating concentrations of inosine or 2'-deoxyinosine were used (Fig. 6).

Thus, for the calf spleen enzyme, it appears that 2'-deoxyinosine causes a greater conformational response than inosine, and this property may be reflected in the non-linear double-reciprocal plots observed with this enzyme when 2'-deoxyinosine, but not inosine, is the variable substrate, as described in the preceding paper (Savage & Spencer, 1979).

Discussion

The results of quantitative differential-inactivation studies reported in the present paper indicate that the fraction of the enzyme that is stabilized by inosine is a function of the concentration of this substrate and is apparently independent of the method of inactivation used. A likely mechanism that would account for this observation is a conformational rearrangement, induced by the binding of the substrate, which alters the accessibility of the relevant peptide bonds to the proteolytic enzymes. As pointed out by Zyk *et al.* (1969), the alternative explanation of shielding effects is rendered unlikely if the rates of differential inactivation by proteinases differing in specificity are affected by a given substrate concentration to the same extent. Furthermore, when the additional independent criterion of thermal inactivation was investigated, the rate of differential inactivation was affected by a given substrate to the same extent as previously observed when using differential proteolysis.

Fig. 5. Effect of inosine and hypoxanthine on the rate of inactivation of rabbit erythrocyte purine nucleoside phosphorylase by 5,5'-dithiobis-(2-nitrobenzoic acid)

The enzyme (60 μ g/ml in 50mM-Tris/HCl buffer, pH 7.5, specific activity 25 units/mg) was incubated with (a) 2.0mM- or (b) 0.02mM-5,5'-dithiobis-(2-nitrobenzoic acid) at 25°C in a final volume of 0.3 ml in the presence of saturating concentrations (2.0mM) of either inosine (Δ) or hypoxanthine ($\bullet$), or in the absence of substrate ($\circ$) as indicated below. Samples (2 μ l) were removed after various time intervals and immediately assayed for purine nucleoside phosphorylase activity. Abbreviation used: DTT, dithiothreitol.

Fig. 6. Effect of inosine and 2'-deoxyinosine on the rate of inactivation of calf spleen and rabbit erythrocyte purine nucleoside phosphorylase by Pronase

(a) The calf spleen enzyme (concentration 30 μ g of protein/ml of 50mM-Tris/HCl buffer, pH 7.5) or (b) the rabbit erythrocyte enzyme (concentration 20 μ g of protein/ml of 50mM-Tris/HCl buffer, pH 7.5) in a final volume of 0.3ml was incubated with Pronase (5 μ g) in the presence of a saturating concentration (0.1 mM) of either inosine or 2'-deoxyinosine, or in the absence of substrates (control) as indicated. Experimental details are described in the text.

The finding that binding of substrate increases the rate of inactivation by 5,5'-dithiobis-(2-nitrobenzoic acid) provides further evidence of a conformational response to the binding of the substrate; it is difficult to envisage a mechanism for this phenomenon without postulating a change in the conformation of the enzyme. It therefore seems likely that conformational changes induced by the binding of substrate may result in a change in the spatial arrangements of thiol groups, which become more accessible for reaction with this reagent.

Substrate-induced conformational changes in purine nucleoside phosphorylase may alter the binding properties of the enzyme in a kinetically significant way. Thus the extent of the conformational response may be reflected in the velocity of the catalytic reaction and may provide a feasible explanation for the apparent negative co-operativity reported for this enzyme in the preceding paper (Savage & Spencer, 1979) and elsewhere (Savage & Spencer, 1977). Observations with calf spleen purine nucleoside phosphorylase may be pertinent in this respect; a consistent relationship was obtained between the conformational response to the binding of 2'-deoxyinosine and the non-linear kinetic plots obtained with this substrate. The fact that inosine does give some protection against inactivation by Pronase and heat for calf spleen purine nucleoside phosphorylase, albeit not to the same extent as 2'-deoxyinosine, indicates that this enzyme may also respond to the binding of inosine with changes in the conformation of the enzyme structure. Such changes may be large enough to influence the stability of the enzyme, but are apparently insufficient to produce non-linear double-reciprocal plots when inosine is the variable substrate.

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