Stimulation of Adenine Phosphoribosyltransferase by Adenosine Triphosphate and other Nucleoside Triphosphates

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1. Adenine phosphoribosyltransferase was protected from inactivation on heating at 55° by the presence of 5-phosphoribosyl pyrophosphate. ATP, adenine, AMP or GMP had no protective effect on the activity of this enzyme. The presence of either 5-phosphoribosyl pyrophosphate or ATP did not protect adenine phosphoribosyltransferase against the loss of ATP stimulation obtained by heating at 55°. 2. At pH 5.3 and 6.0 adenine phosphoribosyltransferase was stimulated by a narrow range of ATP concentration (15–25 μM). At pH 6.5 and 7.0 maximum stimulation was obtained with 25–30 μM -ATP, and at pH 7.4, 8.2 and 8.85 maximum stimulation was obtained over a wide range of ATP concentrations (60–200 μM). With extracts that had been heated for 30 min. at 55° no stimulation was observed at either pH 5.3 or 7.4 with ATP concentrations up to 100 μM . 3. Short periods of heating at 55° (1, 2 or 5 min.) increased the stimulation of adenine phosphoribosyltransferase obtained with various concentrations of ATP. 4. The addition of CTP, GTP, deoxy-GTP, deoxy-TTP or XTP to assay mixtures resulted in weak stimulation of adenine-phosphoribosyltransferase activity. 5. It is suggested that there are at least three different forms of adenine phosphoribosyltransferase, each with a different affinity for ATP.

It has recently been shown that concentrations of ATP less than about 250 μM stimulate the activity of adenine phosphoribosyltransferase in crude extracts of Ehrlich ascites-tumour cells (Murray, 1967). Higher concentrations of ATP were found to inhibit adenine phosphoribosyltransferase. It was suggested that stimulation and inhibition of adenine phosphoribosyltransferase by ATP may be important mechanisms for regulating the activity of this enzyme *in vivo*.

In the present paper the nature of the stimulation of adenine phosphoribosyltransferase by ATP are reported in more detail. Experiments carried out at different pH values have suggested that the enzyme can exist in different conformational states that have different affinities for ATP. Effects similar to those observed with ATP were obtained with a range of ribo- and deoxyribo-nucleoside triphosphates.

EXPERIMENTAL

Substrates and activators

Samples of radioactive and non-radioactive purines used were similar in purity to those described by Murray (1966a).

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The magnesium salt of PRPP* was obtained from the Sigma Chemical Co. (St Louis, Mo., U.S.A.) and was not purified further before use. The purity of the PRPP sample was determined in assays of adenine phosphoribosyltransferase (see below) containing an excess of adenine (62 m μmoles) and a calculated concentration of PRPP (1–5 m μmoles). After incubating for 15 min. at 25°, the reaction had gone to completion and the amount of AMP formed was a measure of the amount of PRPP originally added (see also Henderson & Khoo, 1965). The commercial sample of PRPP was found to be 70% pure.

All samples of ribo- and deoxyribo-nucleoside triphosphates were obtained from the Sigma Chemical Co.

Assays of adenine-phosphoribosyltransferase activity

Assay with DEAE-cellulose disks. Extracts were prepared from Ehrlich ascites-tumour cells after 7–9 days of tumour growth as described by Murray (1966b). Conditions of the assay with [8-¹⁴C]adenine by using DEAE-cellulose disks have been described by Murray (1966a). All assays were carried out at 25°. In studies at different pH values a composite buffer of tris and histidine was used. Assay mixtures contained a final concentration of 50 μM of both tris and histidine.

Separation of products by paper chromatography. Assays were carried out as described above at 25°. After 2 min.,

* Abbreviation: PRPP, 5-phosphoribosyl pyrophosphate.

0.025 ml. samples were removed, spotted directly on to Whatman 3MM paper and dried in a cold air-stream. The chromatograms, containing AMP, ADP and ATP as marker compounds, were developed with isobutyric acid-aq. 0.19 N-NH₃ (661:339, v/v) as solvent. After the papers had been dried, areas corresponding to AMP, ADP and ATP were cut out and radioactivity was measured by direct liquid-scintillation counting as described by Murray (1966b).

RESULTS

Isolation of the nucleotide products of the adenine-phosphoribosyltransferase reaction in the presence and absence of ATP. The products of the adenine-phosphoribosyltransferase reaction (at pH 7.4) in the presence and absence of ATP were separated by paper chromatography in isobutyric acid-aq. ammonia (see the Experimental section). In the absence of ATP the only detectable product was AMP, but in the presence of 60 μ M-ATP considerable radioactivity was associated with ADP and ATP as well as AMP on the paper chromatograms. In a typical experiment 28% of the radioactivity present in nucleotide products formed from [8-¹⁴C]-adenine was associated with AMP, 24% with ADP and 48% with ATP. Under these conditions the added ATP resulted in an increase of 30–40% in the amount of nucleotide formed from adenine. With the isobutyric acid-aq. ammonia solvent used to separate the reaction products of adenine phosphoribosyltransferase, ATP did not separate from IMP (R_f 0.2). Some animal tissues contain an ATP-activated adenylate deaminase (Cunningham & Lowenstein, 1963), and so it was necessary to fractionate the reaction products in a system that separates IMP and ATP. Assays of adenine-phosphoribosyltransferase activity were carried out as described in the Experimental section and the products separated by chromatography in ammonium sulphate–0.1 M-sodium phosphate (pH 6.8)–propan-1-ol (30:50:1, w/v/v). Areas corresponding to AMP + ADP (R_f 0.28), ATP (R_f 0.36) and IMP (R_f 0.55) were cut from the paper and radioactivity was estimated by liquid-scintillation counting. Negligible activity was associated with IMP when assays contained 60 μ M-ATP, but in the presence of 2 mM-ATP about 5% of the total activity incorporated into nucleotides was associated with IMP. Thus it appears that an ATP-activated adenylate deaminase is present in the soluble fraction of Ehrlich ascites-tumour cells. Assays carried out with 60 μ M-deoxy-TTP or with 50 μ M-CTP resulted in some radioactivity being associated with ADP and ATP, but there was no apparent relationship between the extent of incorporation into ADP and ATP and the degree of stimulation of adenine phosphoribosyltransferase. Zero-order initial velocities were always obtained and it would not be expected that removal of AMP would affect the

velocity of the adenine phosphoribosyltransferase.

Incubation of cell-free extracts for 30 min. at 55°, which abolished ATP stimulation of adenine phosphoribosyltransferase (see below), inactivated the enzyme(s) converting AMP into ADP and ATP. Thus in assays with heated extract AMP was the only nucleotide product formed from adenine and PRPP either in the presence or absence of ATP.

Effect of incubation at 55° on the activity of adenine phosphoribosyltransferase and on its stimulation by ATP. Samples of a crude extract from ascites-tumour cells were incubated for various times at 55° in the presence or absence of either 31 μ M-ATP or 40 μ M-PRPP. The samples were rapidly cooled and 0.025 ml. samples assayed for adenine-phospho-

Fig. 1. Effect of heating at 55° on the activity of adenine phosphoribosyltransferase and on the stimulation of this enzyme by ATP. Samples of a crude extract from Ehrlich ascites-tumour cells were heated in the presence of either 40 μ M-PRPP or 31 μ M-ATP or in the absence of both of these compounds. After various times of heating the samples were rapidly cooled and 0.025 ml. portions assayed for adenine-phosphoribosyltransferase activity at pH 7.8 in the presence and absence of 31 μ M-ATP (see the Experimental section). The PRPP concentration was 9.4 μ M. $\circ$, Control; $\bullet$, control assayed in the presence of 31 μ M-ATP; $\square$, heated in the presence of 40 μ M-PRPP; $\blacksquare$, heated in the presence of 40 μ M-PRPP, assayed in the presence of 31 μ M-ATP; $\triangle$, heated in the presence of 31 μ M-ATP; $\blacktriangle$, heated in the presence of 31 μ M-ATP, assayed in the presence of 31 μ M-ATP.

ribosyltransferase activity at 25° in the presence and absence of 31 μM -ATP. The concentration of PRPP in the assay mixtures was 9.4 μM . The results are shown in Fig. 1. It is apparent that the presence of PRPP during the incubation period largely prevented a loss in activity, but had no effect on the gradual removal of the stimulatory effect of ATP. Incubation with 5 μM -adenine, 40 μM -AMP or 480 μM -GMP had no protective effect on the loss of adenine-phosphoribosyltransferase activity during incubation at 55°.

Effect of pH on the stimulation of adenine phosphoribosyltransferase by ATP. Assays were carried out at ATP concentrations between 5 and 200 μM at pH 5.3, 6.0, 6.5, 7.0, 7.4, 8.2 and 8.85.

Fig. 2. Effect of pH on the stimulation by ATP of adenine-phosphoribosyltransferase activity in a crude extract from Ehrlich ascites-tumour cells. Assays were carried out at pH 5.3 (○), pH 6.0 (●), pH 6.5 (△), pH 7.0 (▲), pH 7.4 (□), pH 8.2 (■) and pH 8.85 (▽) in the presence of various concentrations of ATP. The composite buffer system used in these assays is described in the Experimental section.

Fig. 3. Effect of ATP on the activity of adenine phosphoribosyltransferase in a crude extract from Ehrlich ascites-tumour cells that had been heated for 30 min. at 55° in the presence of 40 μM -PRPP. Assays were carried out at pH 5.3 (○) and at pH 7.4 (●).

Assays were also carried out at pH 5.3 and 7.4 on an extract that had been incubated at 55° for 30 min. in the presence of 40 μM -PRPP. The results of these experiments are shown in Figs. 2 and 3. At pH 5.3 and 6.0, adenine phosphoribosyltransferase was only stimulated by a narrow range of ATP concentration (15–30 μM) and the stimulatory effect of ATP was small at higher or lower concentrations. At pH 6.5 and 7.0 maximum stimulation was obtained with 25–30 μM -ATP, and at pH 7.4, 8.2 and 8.85 stimulation was obtained over a wide range of ATP concentrations (60–200 μM). In addition, at the higher pH values (7.4, 8.2 and 8.85) maxima were apparent that nearly corresponded to the ATP concentrations giving maximum stimulation at the lower pH values (5.3, 6.0, 6.5 and 7.0). Maximum stimulation of adenine phosphoribosyltransferase (220%) was obtained at pH 5.3 with 15 μM -ATP. At pH 6.0 maximum stimulation was 210% (15 μM -ATP), at pH 6.5 it was 73% (30 μM -ATP), at pH 7.0 it was 110% (30 μM -ATP), at pH 7.4 it was 39% (80 μM -ATP), at pH 8.2 it was 50% (60 μM -ATP) and at pH 8.85 it was 95% (100 μM -ATP). The stimulation obtained with a concentration of ATP giving maximum stimulation at a particular pH value varied widely with different extracts from ascites-tumour cells, although the shape of the graphs of activity plotted against ATP concentration remained the same. Thus at pH 7.4 the stimulation obtained with 80 μM -ATP in the presence of 9.4 μM -PRPP ranged from 22 to 55%. With heated extracts no stimulation was observed at either pH 5.3 or 7.4 with ATP concentrations up to 100 μM (see Fig. 3).

The effect of ATP on the activity of adenine

Fig. 4. Effect of ATP concentration on the activity of adenine phosphoribosyltransferase in a crude extract from Ehrlich ascites-tumour cells heated for various times at 55°. Extracts were tested for enzymic activity at pH 7.4 in the presence of various concentrations of ATP after heating at 55° for 0 min. (○), 5 min. (●), 10 min. (△) and 15 min. (▲).

phosphoribosyltransferase at pH 7.4 after heating for 0, 5, 10 or 15 min. at 55° is shown in Fig. 4. After heating for 5 min. there was an increase in the stimulation obtained with 35–45 μM-ATP (compared with the control) and a decrease in the stimulation obtained with other ATP concentrations. However, after heating for 10 or 15 min. the shape of the curve was similar to that of the control, but the extent of the stimulation was progressively decreased. In a further experiment, heating extracts for 1 or 2 min. at 55° increased the stimulation obtained with 5–25 μM-ATP by 19–34% (compared with the control), with no effect on the stimulation obtained with 35–45 μM-ATP.

Effect of pH and ATP on the kinetic parameters of adenine phosphoribosyltransferase. The Michaelis constant, K_m , of PRPP for adenine phosphoribosyltransferase decreased as the pH was lowered. Thus the K_m was 13.0 μM at pH 8.85, 7.3 μM at pH 7.8, 5.8 μM at pH 7.0 and 3.8 μM at pH 5.3. Values of the extrapolated maximum velocity were 3.7, 4.2, 4.0 and 3.0 mμmoles of nucleotide formed/min./mg. of protein at pH 8.85, 7.8, 7.0 and 5.3 respectively. Similar results were obtained with adenine phosphoribosyltransferase in extracts that had been desensitized to the stimulatory effect of ATP by incubating for 30 min. at 55°.

The effect of ATP on the kinetic parameters of adenine phosphoribosyltransferase was found to vary in different extracts from ascites-tumour cells. Previous studies had indicated that ATP increased the maximum velocity but did not affect its affinity for PRPP (Murray, 1967). In the present studies ATP increased the maximum velocity of the reaction (about threefold) in three determinations with two different ascites-cell extracts. The K_m

of PRPP with adenine phosphoribosyltransferase was decreased in the presence of ATP (by about 50%) in nine determinations made with four different ascites-cell extracts. All determinations were carried out with freshly prepared extracts, and the varying results are, at present, unexplained.

Inhibition of adenine phosphoribosyltransferase by AMP was competitive with PRPP over the pH range 5.3–8.85 (see Murray, 1966b, 1967). In addition, the inhibitor constant for AMP was approximately equal to K_m over this pH range.

Stimulation of adenine phosphoribosyltransferase by purine and pyrimidine nucleoside triphosphates. Each of the nucleoside triphosphates CTP, GTP, deoxy-GTP, deoxy-TTP and XTP stimulated the activity of adenine phosphoribosyltransferase in freshly made extracts from ascites-tumour cells. When assays were carried out at pH 7.4, plots of activity against triphosphate concentration were similar in shape to those obtained with ATP at this pH (see Fig. 2). Maximum stimulation with CTP, deoxy-GTP, deoxy-TTP and XTP was 44, 20, 24 and 21% respectively and was obtained with a concentration of 70 μM of each nucleotide. A maximal stimulation of 15% was obtained with 40 μM-GTP. The PRPP concentration in all assay mixtures was 22 μM. Each of the triphosphates decreased the K_m of PRPP with adenine phosphoribosyltransferase without affecting the maximum velocity of the reaction. Thus 60 μM-CTP, -deoxy-GTP, -deoxy-TTP and -XTP and 40 μM-GTP decreased the K_m of PRPP by 33, 23, 40, 23 and 19% respectively at pH 7.4. Sufficient experiments were not carried out to determine whether these compounds could also affect the maximum velocity of the adenine-phosphoribosyltransferase reaction under some conditions, as described above for ATP.

The effect of GTP on adenine-phosphoribosyltransferase activity varied considerably when fresh ascites-tumour-cell extracts were not used. In some extracts that had been stored at –15° and thawed and frozen several times GTP did not stimulate the enzymic activity. The stimulatory effects of the other triphosphates, including ATP, were not altered by the time of storage at –15° or by the number of times an extract was frozen and thawed.

When assays were carried out at pH 7.4 with a fresh extract from ascites-tumour cells the combination of ATP with an equimolar concentration of either CTP or GTP resulted in a stimulation of adenine-phosphoribosyltransferase activity less than that obtained with the same concentration of ATP tested alone. In addition, none of the triphosphates had any stimulatory effect on enzyme that had been desensitized to ATP stimulation by heating. The triphosphates did not stimulate adenine phosphoribosyltransferase assayed in the presence of an inhibitory concentration of ATP.

The concentration of ATP required to inhibit the adenine transferase at all (in the presence of $9.4\text{ }\mu\text{M}$ -PRPP) varied between 300 and $500\text{ }\mu\text{M}$ with different batches of extracts from ascites-tumour cells. In the presence of $9.4\text{ }\mu\text{M}$ -PRPP the activity was inhibited by about 33% by 2 mM -ATP and by about 58% by 5 mM -ATP. Berlin & Stadtman (1966) reported that adenine-phosphoribosyltransferase activity in an extract of *Bacillus subtilis* was completely inhibited by 1 mM -ATP in the presence of $250\text{ }\mu\text{M}$ -PRPP. Thus it appears that there are substantial differences between the bacterial and tumour-cell enzymes.

DISCUSSION

In these studies adenine-phosphoribosyltransferase activity in extracts from Ehrlich ascites-tumour cells was protected against thermal inactivation by PRPP (see also Hori & Henderson, 1966). Similar protection of other enzymes has been obtained with their substrates, presumably resulting from stabilization after binding of the substrate at the active centre (for references see Grisolia, 1964). Hori & Henderson (1966) found that $5\text{ }\mu\text{M}$ -adenine and $100\text{ }\mu\text{M}$ -AMP protected the activity of partially purified adenine phosphoribosyltransferase from Ehrlich ascites-tumour cells by 18 and 88% respectively when the enzyme was heated for 10 min. at 40° . In the present experiments $5\text{ }\mu\text{M}$ -adenine and $40\text{ }\mu\text{M}$ -AMP had no protective effect on adenine-phosphoribosyltransferase activity in ascites-tumour-cell extracts incubated at 55° over a 25 min. period. The results are consistent with the mechanism of adenine phosphoribosyltransferase proposed by Hori & Henderson (1966) and implied by the results of Stonehill & Balis (1965), which provides that adenine cannot combine with the enzyme in the absence of PRPP. It is surprising, however, that the competitive inhibitor AMP (see Murray, 1966b) did not protect against thermal inactivation, as this compound would be expected to bind at the active centre of the enzyme. The purified enzyme used by Hori & Henderson (1966) was considerably more heat-labile than the adenine phosphoribosyltransferase in the crude cell-free extracts described in the present paper. Thus heating the purified enzyme for 10 min. at 40° resulted in about 50% loss of activity (Hori & Henderson, 1966), whereas in the present experiments heating a crude extract for 10 min. at 55° resulted in 28% loss of activity. In addition, the catalytic and regulatory properties of adenine phosphoribosyltransferase appear to be modified on purification (Murray, 1967).

The effect of pH on the ATP stimulation of adenine phosphoribosyltransferase could be explained by postulating that at least three different

forms of that part of the enzyme involved in binding ATP exist, each with a different, pH-dependent, affinity for ATP. The forms binding ATP strongly between pH 5.3 and 6.0 and between pH 6.5 and 7.0 showed maximum stimulation at $15\text{ }\mu\text{M}$ - and $30\text{ }\mu\text{M}$ -ATP respectively. In each case, before this maximum was reached, the plots of activity against ATP concentration showed an apparent co-operative effect of ATP. According to the model proposed by Monod, Wyman & Changeux (1965), with systems in which an effector increases the affinity of the enzyme for the substrate, the substrate (in this case PRPP) should show a co-operative interaction with the effector. Plots of activity against PRPP concentration were always hyperbolic either in the presence or absence of ATP (see Murray, 1967). Trant & Lipman (1963) reported that glucose 6-phosphate increased the affinity of glycogen synthetase for UDP-glucose, but found no evidence of a co-operative interaction with substrate either in the presence or absence of the activator. However, in the present experiments an increased affinity of adenine phosphoribosyltransferase for PRPP in the presence of ATP was not always obtained. The extent of the stimulation decreased rapidly as the ATP concentration was increased above that resulting in maximum stimulation. This could be interpreted as a 'substrate-inhibition' type of effect, although such an effect would not be expected to produce such a sharp decrease in stimulation. The results could not be due to ATP inhibition gradually balancing ATP stimulation as, after the sharp decrease from the maximum, the activity remained constant as the ATP concentration was increased further. In contrast, the stimulation occurring between $60\text{ }\mu\text{M}$ - and $200\text{ }\mu\text{M}$ -ATP which predominated between pH 7.4 and 8.85 did not show a clearly defined maximum concentration. The decrease in stimulation that did occur at pH 7.4 as the ATP concentration was increased may be due to the increasing influence of ATP acting as an inhibitor.

It is noteworthy that heating crude extracts from ascites-tumour cells for short periods at 55° increased the stimulation by various concentrations of ATP. This suggests that the different forms of the ATP-binding portion of the native enzyme may exist in a constrained state that restricts their ability to modify the catalytic properties of the active site. Short periods of heating may release this constraint inherent in the native enzyme molecule, enabling an increase in the degree of stimulation obtained with a given concentration of ATP.

The physiological significance of these findings is difficult to assess. The extent of stimulation of adenine-phosphoribosyltransferase activity by nucleoside triphosphates or of its inhibition by ATP

and AMP will depend on the intracellular concentrations of these nucleotides and of PRPP. ATP concentrations in Ehrlich ascites-tumour cells of $3\mu\text{moles/g.}$ of packed cells (Klenow, 1962) and of $4.8\mu\text{moles/g.}$ of packed cells (Overgaard-Hansen, 1965) have been reported. Henderson & Khoo (1965) estimated that ascites-tumour cells contained about $0.3\mu\text{mole}$ of PRPP/g. of packed cells. If each compound were evenly distributed throughout the cell, and assuming an inhibitor constant for ATP of $395\mu\text{M}$ (Murray, 1966b), adenine-phosphoribosyltransferase activity would be inhibited by about 10%. Therefore, if the mechanism that exists for stimulation of adenine phosphoribosyltransferase is to have any physiological significance, it is necessary to postulate 'compartmentation' within the adenine nucleotide pool and within the PRPP pool of ascites-tumour cells. There is already some evidence of 'compartmentation' within the adenine nucleotide pool of Ehrlich ascites-tumour cells (Murray, 1966b; Kandaswamy & Henderson, 1962) and of bacterial cells (Berlin & Stadtman, 1966). It is significant that ATP can stimulate the adenine phosphoribosyltransferase in the presence of the competitive inhibitor AMP to an extent dependent on the AMP concentration (Murray, 1967). Thus control of the activity of adenine phosphoribosyltransferase in Ehrlich ascites-tumour cells may

largely depend on the relative local concentrations of AMP, ATP and PRPP *in vivo*.

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