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DIADENOSINE TETRAPHOSPHATE ACTIVATES AMP DEAMINASE FROM RAT MUSCLE

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SUMMARY: Diadenosine tetraphosphate, Ap_4A, doubled the activity of AMP deaminase from rat muscle, with an activation constant of 0.005 mM, in the presence of 0.05 mM AMP. The presence of Ap_4A appeared to induce Michaelian kinetic behavior. The activation by Ap_4A was not dependent on the presence of either $MgCl_2$ or KCl in the reaction mixture. Diguanosine tetraphosphate was inhibitor of the enzyme. Diadenosine and diguanosine triphosphates, adenylosuccinate and xanthosine monophosphate were neither inhibitors nor activators of the reaction.

The interest in the potential regulatory role of diadenosine 5',5'''-P^1,P^4-tetraphosphate (Ap_4A) has increased in the last years (1-5). A regulatory effect of diguanosine 5',5'''-P^1,P^4-tetraphosphate (Gp_4G) on the interconversion of purine nucleotides has been described (6); the possibility that Ap_4A and Gp_4G could have in some cases complementary physiological actions has also been raised (7). A possible example of this kind of effect is presented in this paper.

Gp_4G is present at high concentrations in *Artemia* gastrulae and serves as a source of adenine and guanine nucleotides during development of larvae (8). In this crustacean Gp_4G is an activator, at nanomolar concentrations, of GMP reductase thus favoring its transformation to IMP and subsequently to AMP (Fig.1). GMP reductase from calf thymus and *Artemia* are also inhibited by XMP (6,9). Similar effects of Gp_4G and XMP have also been described for the reductase from erythrocytes (10).

Based both on the above results and on the apparent symmetry (Fig.1) of the purine nucleotide interconversion

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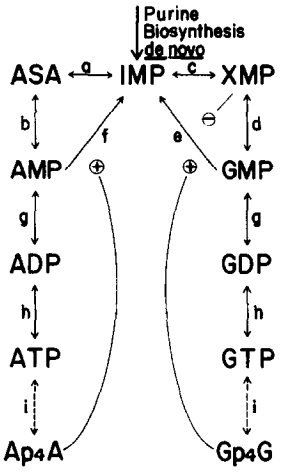

Figure 1. Purine nucleotide interconversion. The enzymes acting in this pathway are the following: a, adenylosuccinate synthetase (EC 6.3.4.4); b, adenylosuccinate lyase (EC 4.3.2.2); c, IMP dehydrogenase (EC 1.2.1.14); d, GMP synthetase (glutamine-hydrolyzing) (EC 6.3.5.2); e, GMP reductase (EC 1.6.6.8); f, AMP deaminase (EC 3.5.4.6); g, nucleosidemonophosphate kinase (EC 2.7.4.6); i, this pathway is still not well known. Dinucleosidetetraphosphatase (EC 3.6.1.17) splits both Ap_4A and Gp_4G to the corresponding nucleosides mono and triphosphates.

pathway, it seemed to us of interest to test the effect of Ap_4A and adenylosuccinate (ASA) on AMP deaminase from rat muscle. Whereas, in our experimental conditions, ASA did not inhibit the enzyme, Ap_4A was an activator of the deaminase.

MATERIALS AND METHODS

Purification of the enzyme

Female white rats fed ad libitum were used through these experiments. The enzyme was purified essentially as described by Coffee and Kofke (11). Part of the legs and back muscles (8 g) were excised, cut into small pieces and homogenized in a meat grinder with 30 ml of 0.1M potassium phosphate buffer, pH 6.5, 0.18 M KCl and 2 mM 2-mercaptoethanol (buffer A). The brei was stirred at room temperature for 1 h, centrifuged at 50,000 x g for 30 min and the supernatant filtered through glass wool. Part (23.5 ml) of the total extract (31 ml) was mixed with a slurry of 20 ml of phosphocellulose type P, (0.64 meq/g from Serva; around 1.4 g of cellulose resuspended up to 20 ml with buffer A), stirred for 30 min at room temperature and centrifuged at 20,000 x g for 15 min. The supernatant was discarded and the precipitate washed 3 times with 20, 20 and 10 ml of buffer A. The final precipitate was packed into a column (5.3 x 1.2cm) which was washed, until the absorbance at 280 nm of the eluate was near zero, with 20 ml of 0.45 M KCl, 2 mM 2-mercaptoethanol (adjusted to pH 7.0 with K_2HPO_4). The enzyme was eluted with a linear gradient consisting of 30 ml of 0.45 M KCl, 2 mM 2-mercaptoethanol (pH 8.0) and 30 ml of 1.5 M KCl, 2 mM 2-mercaptoethanol (pH 8.0). Fractions of 1.2 ml were collected. Fractions 4-11 with maximum enzyme activity were pooled (9.6 ml). An aliquot (7 ml) of this pool was dialyzed over-

night against 1 l of 0.045 M potassium phosphate buffer pH 7.2, 2 mM 2-mercaptoethanol, and applied to a DEAE-cellulose column (2.5 x 1.5) equilibrated with the dialysis buffer. The column was washed with 7 ml of the same buffer and the enzyme eluted with a linear gradient of 15 ml of 0.045 M potassium phosphate , 2 mM 2-mercaptoethanol (pH 7.2) and 15 ml of 0.45 M potassium phosphate, 2 mM 2-mercaptoethanol (pH 7.2). Fractions of 1 ml were collected; the enzyme eluted in fractions 3,4 and 5 comprising a volume of 3 ml. This pool had 420 mU/ml of AMP deaminase activity and 0.038 mg protein/ml.

Enzymatic assay

Unless otherwise stated the reaction mixture contained in a final volume of 1 ml the following components: 50 mM imidazole-HCl pH 6.5, 0.1 M KCl, 0.050mM AMP. The reaction was initiated by addition of the enzyme. Initial velocities were measured following decrease in absorbance at 265 nm ($\Delta\varepsilon_{mM} = 8.86$) or increase in absorbance at 285 nm ($\Delta\varepsilon_{mM} = 0.23$) in cuvettes of 1-cm light path (12).

Other materials

Diguanosine tri and tetraphosphate were obtained from Artemia cysts as previously described (13); the other nucleotides were purchased from Sigma.

RESULTS

The effect of increasing concentrations of Ap_4A on the initial velocity of the reaction catalyzed by AMP deaminase, at a fixed (0.05 mM) AMP concentration is presented in Fig.2. The maximal activation of the enzyme, accomplished at a concentration of 0.03 mM Ap_4A, was about four-fold in relation to a control without Ap_4A. Higher concentrations of this nucleotide were inhibitory. The calculated activation constant for Ap_4A was about 5 μM.

The result of Fig.2 represents a true activation of the enzyme for the following reasons. Ap_4A was not a substrate of the reaction. The velocity obtained when Ap_4A was tested

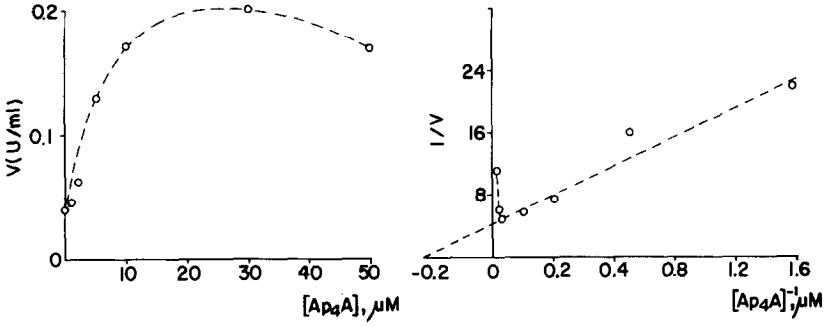

Figure 2. Activation of AMP deaminase by diadenosine tetraphosphate. The concentration of AMP in the reaction mixture was kept at 0.05mM in all cases. The concentrations of Ap_4A were as indicated in the figure. The enzyme was from the P-cellulose step. The reaction was followed at 265nm.

as substrate of the reaction at concentrations of up to 0.02 mM was less than 1% of that obtained in the presence of 0.05 mM AMP. On the other hand it was known that AMP deaminase could be inactivated at low concentration of the enzymatic protein in the reaction mixture (14,15). It seemed then of interest to test whether Ap_4A could actually protect the enzyme during the time of the assay. For that, 5 µl aliquots of the enzyme preparation from the P-cellulose step were incubated in the usual assay conditions, in the absence and presence of 0.0125 mM Ap_4A. After 5,10,15 and 20 min at 37ºC, the reaction was started by the addition of AMP (0.05 mM) and, when appropriate, supplemented with Ap_4A. At those times, the measured initial velocities were 71,53,43 and 33 per cent of that observed in an unincubated control, regardless of whether Ap_4A was present throughout, or added at the end of the incubation. These results indicate that the effect of Ap_4A on the deaminase represents activation and not protection of the enzyme.

The effect of fixed (6.3 µM) concentrations of Ap_4A on AMP deaminase is presented in Fig.3. In our experimental

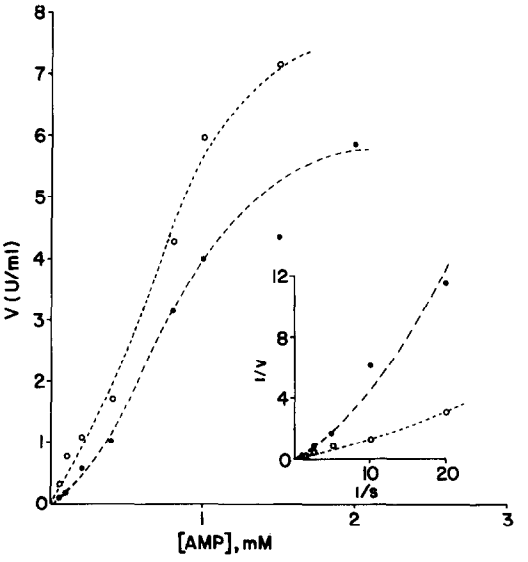

Figure 3. Effect of AMP concentration, in the presence or absence of diadenosine tetraphosphate, on the initial velocity of AMP deaminase. The concentration of Ap_4A was 0.0063mM (o). The enzyme was from the DEAE-cellulose step. The reaction was followed at 285nm.

TABLE I

Effect of various nucleotides on the activity of AMP deaminase

Nucleotide added (0.025 mM)	velocity (%)
-	100
Ap_4A	234
Gp_4G	20
Ap_3A	109
Gp_3G	82
ATP	37
GTP	22
XMP	100
Adenylosuccinate	109

The reaction was followed at 265nm in the presence of 0.05 mM AMP. When indicated, other nucleotides were tested as effectors, all of them at 0.025mM concentration. The enzyme used was from the DEAE-cellulose step.

conditions the enzyme transforms AMP with a kinetics suggestive of positive cooperativity. In the presence of Ap_4A the Lineweaver-Burk plots approached linearity. The effect of Ap_4A on the enzyme, compared with that of other nucleotides, all of them at around 0.025 mM concentration, is presented in Table I. Ap_4A was an activator of the reaction, but other dinucleoside polyphosphates analogs behaved differently. Whereas Ap_3A and Gp_3G did not affect the velocity, Gp_4G was an inhibitor of the reaction. As previously known, ATP and GTP were inhibitors of rat muscle enzyme (12,16). ASA and XMP did not affect the velocity of the reaction in our experimental conditions.

Finally the effect of Ap_4A was also assayed in the absence and presence of increasing concentrations of either $MgCl_2$ or KCl. Concentrations of up to 10 mM $MgCl_2$ or 0.1 M KCl did not change the activation (around two fold) accomplished by 0.012 mM Ap_4A in the presence of 0.1 mM AMP.

DISCUSSION

Ap_4A and Gp_4G appear to have a role in the control of purine nucleotide interconversion. Gp_4G activates GMP reductase and, consequently favors the transformation of guanine into adenine nucleotides. The physiological importance

of this is apparent during development of the Artemia encysted gastrula to free swimming larva; in this period Gp_4G serves both as a source of adenine nucleotides (8) and as a positive effector of the transformation of the GMP moiety of the tetraphosphate into IMP. The results here presented show that Ap_4A is activator of AMP deaminase and, by a similar token, may favor the transformation of adenine into guanine nucleotides.

Details of the effect of both tetraphosphates on the purine nucleotide interconversion are, however different. Half activation of the reductase and deaminase by Gp_4G and Ap_4A occurs at concentrations in the order of nM and μM, respectively. Although the kinetic behaviors of both enzymes are different the effects of both Gp_4G and Ap_4A tend to induce Michaelian kinetics. The effect of Gp_4G on the reductase is mimicked by higher concentrations of GTP (6,10), but ATP and Ap_4A have opposite effects on rat muscle deaminase. In our experimental conditions, Ap_4A did not have an appreciable effect on the initial velocity of GMP reductase (6) and Gp_4G was an inhibitor of AMP deaminase. In addition XMP is a potent inhibitor of the reductase (6,9,10) and ASA is not inhibitor of the deaminase. Finally, it is also worthy to recall that AMP deaminase from different sources may present different regulatory properties (16,17,18,19). In summary, whereas the synthesis of IMP from both GMP and AMP is stimulated by Gp_4G and Ap_4A, respectively, the regulation of the nucleotide interconversion pathway seems to result from a complex array of mechanisms not necessarily of a symmetrical character. The elucidation of the exact roles of Ap_4A and Gp_4G in the control of this pathway deserves further work.

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