Modification by Liposomes of the Adenosine Triphosphate-Activating Effect on Adenylate Deaminase from Pig Heart

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Adenylate deaminase (AMP deaminase, EC 3.5.4.6) of a high substrate specificity was purified from pig heart by chromatography on cellulose phosphate. The enzyme shows a co-operative binding of AMP [h (Hill coefficient) 2.35, with $s_{0.5}$ (half-saturating substrate concentration) 5 mM]. ATP and ADP act as positive effectors, lowering h to 1.55 and $s_{0.5}$ to 1 mM. The addition of liposomes (phospholipid bilayers) to ATP-activated or ADP-activated enzyme causes a further shift of the h value to 1.04 and $s_{0.5}$ to 0.5 mM. For ATP-activated enzyme the addition of liposomes increases V_{max} by about 100%, and for ADP-activated enzyme by 50%. Liposomes have no effect on the kinetics of AMP deaminase in the absence of ATP and ADP, and neither do they influence the inhibitory effect of orthophosphate on heart muscle AMP deaminase. Metabolic implications of these findings are discussed.

The purine nucleotide cycle (Lowenstein, 1972) is a metabolic pathway of special importance for heart physiology (Żydowo, 1976). Adenylate deaminase (EC 3.5.4.6), the NH_3 -forming enzyme in the cycle, has been found in the cytosol of most vertebrate tissues (Purzycka & Żydowo, 1960; Purzycka 1962; Ogasawara *et al.*, 1974, 1975). In skeletal muscle it is partly complexed with myosin (Currie & Webster, 1962; Byrnes & Suelter, 1965; Żydowo *et al.*, 1974; Ashby & Frieden, 1977). The association of some other enzymes with either natural membranes (Entman *et al.*, 1977) or with artificial phospholipid vesicles (Wooster & Wrigglesworth, 1976) has been shown to influence the catalytic properties of the enzyme. Adenylate deaminase from the heart has been little investigated so far (e.g. Chung & Bridger, 1976), and any factor influencing the regulatory properties of this enzyme might affect the operation of purine nucleotide cycle in the heart. One of the factors influencing either the activity or the regulatory properties of any soluble cytosolic enzyme may be its reversible association with intracellular membranous structures. Liposomes are frequently used as a model that may mimic the effect of natural membranes.

In the present paper it is shown that, although liposomes, the phospholipid-containing model membrane structures, do not influence the non-activated kinetics of adenylate deaminase from pig heart, they increase very strongly the activating effect of ATP and ADP on this allosteric enzyme.

Materials and Methods

Adenosine, AMP, ADP, ATP, adenosine 2'-phosphate, adenosine 3'-phosphate, dAMP, CMP and CTP were purchased from Sigma Chemical Co.,

St. Louis, MO, U.S.A. Cyclic AMP was from Schuchard, München, Germany; cellulose phosphate P-11 was from Whatman, Maidstone, Kent, U.K. Egg phosphatidylcholine (lecithin) was obtained from Serva, Heidelberg, Germany; phosphatidylethanolamine (from egg) and phosphatidic acid (from egg phosphatidylcholine) were from Koch-Light, Colnbrook, Bucks., U.K. Adenosine 5'-phosphoroamidate and adenosine 5'-sulphate A grade were obtained from Calbiochem, San Diego, CA, U.S.A. All other chemicals were of highest analytical grade obtainable from P.O.Ch., Gliwice, Poland.

Enzyme preparation

AMP deaminase from pig heart was purified by cellulose phosphate chromatography essentially as described by Smiley *et al.* (1967) for the skeletal-muscle enzyme, with slight modifications. All the steps were performed at 4°C instead of at room temperature, the extraction was carried out by stirring the homogenate for 12 h, and the adsorption on cellulose phosphate was prolonged for 1 h. The elution profile from cellulose phosphate is shown in Fig. 1. The enzyme obtained was stable for at least 1 month when stored at 4°C, and it showed optimum activity at about pH 6.5 in potassium succinate buffer. It was very specific, no deamination of 2'-AMP, 3'-AMP, cyclic 3':5'-AMP, dAMP, ATP, CMP, CTP, adenosine 5'-phosphoroamidate, adenosine 5'-sulphate or adenosine being detected even when incubated with these compounds for 60 min.

Enzyme assay

The incubation mixture, in a final volume of 0.5 ml, contained 100 mM-potassium succinate buffer,

Fig. 1. Elution profile of pig heart AMP deaminase from a cellulose phosphate column

The heart-muscle extract and phosphocellulose column were prepared as described by Smiley *et al.* (1967). Elution was carried out with 1 M-KCl, pH 7, at 4°C. The flow rate was 10 ml/h; 5 ml fractions were collected. ○, Protein concentration (mg/ml); ●, specific activity (μmol of NH₃/min per mg of protein). From the extract containing 13 200 mg of protein, 3 mg of the purified enzyme was obtained. The purification factor was about 850 and the yield of the enzyme activity 19%.

pH 6.4, 150 mM-KCl, 5–10 μg of enzyme protein, AMP, and other additions as indicated in the Figures. The incubation was carried out for 10 min at 30°C; the reaction was started by the addition of enzyme solution, terminated by the addition of phenol reagent and the NH₃ liberated was determined. About 4–5 mg of protein was contained in the incubation mixture when crude extracts were assayed for enzyme activity. In this case the incubation was terminated by the addition of an equal volume of 15% (w/v) trichloroacetic acid, the solution was neutralized with KOH, and NH₃ was determined in a portion of the clear supernatant.

For the investigation the effect of liposomes, 25–50 μl of enzyme solution containing 5–10 μg of protein was preincubated for 1 h at 4°C with 250 μl of liposome suspension containing 375 nmol of phospholipid phosphorus, potassium succinate buffer and KCl. In this case, the reaction was started by the addition of an appropriate amount of AMP solution,

to make up the final volume of the incubation mixture to 0.5 ml.

Preparation of liposomes

Positively charged, negatively charged and neutral liposomes were prepared essentially as described by Galla & Sackmann (1975). The positively charged liposomes contained phosphatidylcholine and phosphatidylethanolamine in the ratio 13:4 (w/w), the negatively charged liposomes contained phosphatidic acid and phosphatidylcholine in the ratio 4:13 (w/w), and the neutral liposomes contained phosphatidylcholine only. Lipid films were deposited on the surfaces of glass vessels by evaporation, under a stream of N₂, of chloroform solutions containing 22.5 nmol of appropriate phospholipids. Then 15 ml of 100 mM-potassium succinate buffer, pH 6.4, containing 150 mM-KCl was added and sonicated under N₂ at room temperature for 10 min at 24 kHz

with a 100W MSE ultrasonic disintegrator. Before sonication the O_2 concentration of the sample was lowered by bubbling N_2 through the sample for 10min. In this way the O_2 concentration reached about 0.010mM as measured by a Clark-type oxygen electrode. Transparent and only faintly opalescent dispersions have been obtained by this procedure which consist of phospholipid-bilayer structures (Phillips, 1972).

Analytical procedures

NH_3 was measured by the phenol/hypochlorite method described by Chaney & Marbach (1962). Protein was determined either by the method of Lowry *et al.* (1951) or by the method of Warburg & Christian as described by Layne (1957). Phospholipid phosphorus after mineralization was determined as described by Gomori (1942).

Results

In contrast with AMP deaminase from skeletal muscle, which is sensitive to the ATP-activating effect only at low K^+ concentration (Zielke & Suelter, 1971), the enzyme isolated from heart muscle is influenced by both ATP and ADP, even in the presence of 150mM-KCl (Figs. 2a and 2b). As Figs. 2(a) and 2(b) show, the velocity of the reaction increased in a sigmoidal fashion with increasing AMP concentration in the absence of ATP and ADP, both in the presence and absence of liposomes. Addition of ATP or ADP to the reaction mixtures shifted the kinetics from sigmoidal to hyperbolic, thereby lowering the apparent $s_{0.5}$ (half-saturating substrate concentration) for AMP. This value changed from 5mM to about 1.5mM with the addition of 1mM-ATP or -ADP. The addition of liposomes did not influence the enzyme kinetics in the absence of ATP and ADP. However, in the presence of ATP, pre-incubation of the enzyme with liposomes caused a further shift of $s_{0.5}$ to 0.5mM and a 2-fold increase of V_{max} . (Fig. 2a).

From the Hill equation:

$$\log \left(\frac{v}{V_{max} - v} \right) = h \log ([AMP]) - \log K$$

the h (Hill coefficient) value for the plots with AMP was 2.35. With the addition of ATP at 1mM, the h value for the same plots was 1.55, and with the addition of ATP and liposomes it was 1.04. The addition of liposomes to the medium in the absence of ATP and ADP did not change the enzyme kinetics, the h value in this case being 2.41.

Liposomes without charge, positively charged liposomes and negatively charged liposomes all had a pronounced effect on the ATP-activated AMP

deaminase (Table 1). None of the liposomes tested influenced the activity of AMP deaminase in the absence of ATP and ADP (Table 1). The effect of liposomes composed of phosphatidylcholine only (those with no charge) was greatest, and hence the detailed experiments were performed with these liposomes. As little as 75nmol of phospholipid added in the form of liposomes had a considerable effect on the ATP-activated AMP deaminase activity (Fig. 3), and the effect increased with the increasing amount of liposomes added up to 300nmol of phospholipid in the sample.

Although no information is available about the effect of orthophosphate on AMP deaminase from heart, this compound is known to exhibit a negative allosteric effect on AMP deaminase from skeletal muscle and brain (Zielke & Suelter, 1971). One could suspect that liposomes would affect not only the activating effect of ATP but also the inhibiting effect

Fig. 2. Effect of phosphatidylcholine-containing (uncharged) liposomes on the ATP-activated (a) and ADP-activated (b) AMP deaminase from pig heart

For experimental details, see the text. AMP deaminase in: ●, the absence of ATP, ADP and liposomes; ○, the presence of liposomes; △, the presence of 1mM-ATP; ▲, the presence of 1mM-ATP and of liposomes containing 375nmol of phospholipid; □, the presence of 1mM-ADP; ■, the presence of 1mM-ADP and liposomes containing 375nmol of phospholipid.

Table 1. *Influence of ATP and differently charged liposomes on the relative activity of AMP deaminase from pig heart*
The activity was assayed in the presence of 10 mM-AMP. The values represent the mean relative activity $\pm$ s.e.m. Enzyme activity in the absence of both ATP and liposomes is expressed as 100, to which all the other values are related. For experimental details, see the text.

		In the presence of liposomes		
	Without liposomes	With no charge	Negatively charged	Positively charged
AMP	100	112 ± 13	110 ± 15	101 ± 7
AMP+ATP	159±13	263 ± 13	217 ± 7	207 ± 9

Fig. 3. *Influence of the amount of phosphatidylcholine added as liposomes on the activity of unactivated and ATP-activated AMP deaminase from pig heart*

For experimental details, see the text. The open bar represents the control; hatched bars indicate samples containing either liposomes and no ATP (■) or 1 mM ATP and no liposomes (■). Solid bars represent experiments in which both 1 mM-ATP and different amounts of liposomes were present in the sample.

Fig. 4. *Inhibitory effect of orthophosphate on AMP deaminase from pig heart*

For experimental details see the text. ○, Control; ■, 2 mM-phosphate; ●, 5 mM-phosphate; ▲, 10 mM-phosphate.

of orthophosphate on the heart enzyme. P_i was shown to be a potent negative effector of AMP deaminase from pig heart (Fig. 4). Liposomes had no effect on this inhibitory action of phosphate (Fig. 5).

Discussion

The molecular nature of the observed effect of liposomes on the kinetics of heart AMP deaminase may only be the subject of speculation at present. If association of liposomes with the enzyme does occur, it cannot be very strong, since an attempt to separate the complex on Sepharose 4B or by centrifugation failed (results not shown). The mixture of liposomes

and AMP deaminase was separated into two components, only one of which showed enzyme activity, the other containing the liposomes and no AMP deaminase activity. After separation from liposomes on Sepharose 4B, the enzyme again showed the same basal activity and was susceptible to the activating effect of both ATP and liposomes in the same way as before.

On addition of ATP and liposomes, the h value derived from the Hill plot decreased from 2.32 to about 1. The enzyme changes from a form that exhibits co-operative binding of AMP to a form that conforms to Michaelis-Menten kinetics. The h value of 2.35 may indicate a minimum of three co-operative binding sites for AMP (Cornish-Bowden, 1976), and is consistent with the proposed tetrameric structure of the enzyme from skeletal muscle (Coffee & Kofke, 1975; Boosman & Chilson, 1976). A similar shift in the h value of the Hill plots on addition of ATP alone has been observed with the enzyme from calf brain (Setlow & Lowenstein, 1967), rabbit skeletal muscle (Smiley & Suelter, 1967) and inverte-

Fig. 5. Influence of phosphatidylcholine-containing (uncharged) liposomes on the orthophosphate inhibition of AMP deaminase from pig heart

●, 5mM-AMP in the absence of liposomes; ○, 5mM-AMP in the presence of liposomes corresponding to 375nmol of phospholipid; ▲, 10mM-AMP in the absence of liposomes; △, 10mM-AMP in the presence of liposomes corresponding to 375nmol of phospholipid.

brate muscle (Gibbs & Bishop, 1977). It is possible that ATP, while bound to both lipid layer and the heart AMP deaminase, exhibits its activating effect more efficiently.

One is tempted to suppose that an effect similar to that shown by liposomes may be displayed by natural phospholipid-containing membranes in the heart cell. It has been proposed by Berne (1964) that adenosine is a mediating factor in the autoregulation of the coronary blood flow in the heart. Adenosine is produced in the tissue by the action of 5'-nucleotidase on AMP, and hence the abundance or shortage of the last compound is a crucial regulatory factor of adenosine concentration in the tissue (Żydowo, 1976). AMP deaminase, if not inhibited, removes AMP from the adenine nucleotide pool, thus diminishing the concentration of the substrate for 5'-nucleotidase. High activity of AMP deaminase observed in the presence of ATP may occur in the tissue when O₂ supply is sufficient and production of ATP in mitochondria exceeds its hydrolysis in the course of heart myosin adenosine triphosphatase activity. In this case, adenosine as a factor increasing O₂ supply through an increased coronary blood flow is not required and AMP concentration should be kept low. If an excessive orthophosphate production from ATP is taking place as a result of predominance of myosin adenosine triphosphatase action over oxidative phosphorylation, AMP deaminase would be inhibited (Fig. 4), and enhanced adenosine production could occur. Burger & Lowenstein (1967) calculated from their experiments with heart extracts

that the presence of ATP may cause a 5-fold increase of AMP deamination and a 10-fold decrease of adenosine production from AMP.

Supposing that natural membranes (e.g. mitochondrial membrane) would display effects on the kinetics of AMP deaminase similar to those shown by liposomes, one may suspect that the molecules of enzyme that are in close contact with the membrane would be even more susceptible to the changes of ATP concentration. This would speed up the functioning of the subtle interplay of several regulatory factors influencing the activity of AMP deaminase and hence the operation of the whole purine nucleotide cycle as well as adenosine production in the heart. The effect of liposomes described here is peculiar to AMP deaminase from heart, as we were unable to show this effect when using the enzyme from skeletal muscle both at high and low concentrations of K⁺. It is therefore even more tempting to suggest that the physiological significance of the effect of phospholipid-containing membranous structures on AMP deaminase is connected with the speeding up of the regulatory effects so important for heart function.

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