The influence of phosphatidate bilayers on pig heart AMP deaminase

Crucial role of pH-dependent lipid-phase transition

Michał WOŹNIAK, Ewa KOSSOWSKA, Jadwiga PURZYCKA-PREIS and Mariusz M. ŻYDOWO

Department of Biochemistry, Academic Medical School, Dębinki 1, 80-211 Gdańsk, Poland

Phosphatidate bilayers composed of dilauroylphosphatidate, dimyristoylphosphatidate, dipalmitoylphosphatidate and dioleoylphosphatidate were prepared. Their interaction with AMP deaminase isolated from pig heart was investigated. Dioleoylphosphatidate bilayers were found to exert non-competitive inhibition on the AMP deaminase with a K_i of 15×10^{-6} M. This inhibition is three orders of magnitude stronger than that exerted by orthophosphate. The phosphatidate species containing saturated fatty acids were either non-inhibitory or inhibited enzyme activity rather poorly. However, alkalization of the medium from pH 6.5 to pH 7.9 led to the inhibition of pig heart AMP deaminase by dilauroylphosphatidate bilayers. This was accompanied by the fluidization of the saturated phosphatidate species, i.e. the lowering of their phase transition temperature in alkaline pH, as measured by light-scattering and fluorescence scans. The possible significance of these findings for the regulation of AMP deaminase activity *in vivo* by natural membranes is discussed.

INTRODUCTION

Several years ago some glycolytic enzymes, regarded as typically cytoplasmic proteins, were found to associate with cellular membranes [1]. Srere [2] postulated that the enzymes of the Krebs cycle may exist in an ordered array bound to the matrix side of the inner-mitochondrial membrane. Some of these enzymes have been found to change their activity on liberation from the membrane [3,4]. Reversible association of phosphofructokinase with erythrocyte membrane is known to change the essential allosteric and regulatory properties of this enzyme [5]. Glutamate dehydrogenase can bind to acidic phospholipids and to the inner-mitochondrial membrane which makes it a peripheral membrane protein [6]. Specific interaction of this enzyme with acidic phospholipids may also be important for its synthesis in the endoplasmic reticulum [7]. AMP deaminase from pig heart is a cytosolic enzyme which may be the subject of a subtle interplay of various regulatory factors, including purine nucleotides, orthophosphate and lipid membranes. Interaction of this enzyme with phosphatidylcholine-containing membranes was shown to enhance the activating effect of ATP and ADP [8]. Cardiolipin was found to inhibit the activity of AMP-deaminase, whereas a complex mixture of natural phospholipids isolated from heart cells, displayed an activating effect [9].

Temperature-triggered phase transition of liposomes to a more fluid, expanded state, reflecting changes in the fluidity and packing density of the fatty acyl chains, has been observed also in the natural membranes. Investigation of the cell behaviour in an electric field revealed that they migrate towards the anode, which indicates that the cell membrane possesses a negative

net surface charge. Ionizable phosphate groups of the membrane phospholipids are partially responsible for the negative charge. Among the lipids carrying a net negative charge between pH 3 and 9 are cardiolipin, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol and phosphatidic acid [10]. The ordered–fluid-state transition in phosphatidic acid bilayers produces a release of protons which results in the decrease of the bulk pH [11]. This implies that the biomembranes containing acidic phospholipid may create effects connected with local charge and pH changes. A complete titration of phosphatidic acid bilayer membranes revealed profound changes in the transition temperature of the respective phospholipid, followed by the pH change [12]. It is well known that in the membrane function an important role is played not only by structural insoluble proteins, but also by water-soluble proteins which can be bound reversibly to the membranes.

The aim of the experiments presented here was to investigate the interaction of the purified pig heart AMP deaminase with phosphatidic acid bilayers composed of single well-characterized acidic lipid species while changing their properties by temperature and pH. This has been done in the hope of gaining a better understanding of the more complex situations which may occur *in vivo*.

MATERIALS AND METHODS

Chemicals

AMP, ATP, phosphatidic acids, and Mops were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Cellulose phosphate P-11 was from Whatman, Maidstone, Kent, U.K. *N*-Phenyl-1-naphthylamine (NPN) was obtained from Eastman Kodak Co. Stock solutions

Abbreviations used: NPN, *N*-phenyl-1-naphthylamine; DLPA, dilauroylphosphatidate; DMPA, dimyristoylphosphatidate; DPPA, dipalmitoylphosphatidate.

of NPN in methanol were prepared daily and stored in the dark. A methanol content below 3% (v/v) did not influence the lipid-phase transition. Four-times distilled water was used throughout the experiments. All other chemicals were of highest analytical grade obtainable from P.O.Ch., Gliwice, Poland.

Fluorescence measurements

The NPN probe was added to 3 ml aliquots of the phospholipids dispersion. Several temperature scans of the fluorescence intensity (I), were recorded at decreasing or increasing temperature using an Aminco Bowman spectrofluorimeter. The fluorescence signals were corrected for light scattering and inner filter effects; the latter correction was by far the more important because the excitation wavelength of 340 nm for NPN corresponds to the absorption maximum [13]. Correction curves were established by measuring the fluorescence intensity of the probe in water as a function of probe concentration. To equilibrate the system it was important to temper the lipid dispersion in the fluid state, about 5 °C above transition temperature (T_i) for 10 min, before starting the cooling run. The ordered-fluid-phase transition in phosphatidic acid and phosphatidylcholine dispersions was monitored by following the change in the fluorescence [13] and in 90° light scattering at 400 nm [12]. The phase transition is indicated by a large increase in the fluorescence intensity or an abrupt decrease in 90° light-scattering intensity.

Sample preparation

Liposomes containing different kinds of phospholipids were prepared as described previously [8], with slight modifications. A stream of argon instead of nitrogen was used throughout the preparation of liposomes containing dioleoylphosphatidate. Liposomes composed of saturated acyl chain phospholipids were sonicated at a temperature well above transition temperature. The resulting lipid dispersions were freshly prepared every day.

Enzyme preparation

AMP deaminase from pig heart was purified by cellulose-phosphate-affinity chromatography as previously described [8].

Enzyme assay

The incubation mixture, in a final volume of 0.5 ml, contained 100 mM-potassium succinate, pH 6.5, 100 mM-KCl, 10 µg of enzyme protein, AMP, and other additions as indicated in the Figures. In experiments performed at a more alkaline pH, 10 mM-Mops/KOH buffer with 100 mM-KCl instead of succinate buffer was used. The incubation was carried out for 10 min at 35 °C, the reaction was started by the addition of the enzyme solution, terminated by the addition of phenol reagent and the amount of NH_3 liberated was determined. To investigate the effect of liposomes 50 µl of enzyme solution containing 10 µg of protein was preincubated for 20 min at 4 °C with 250 µl of liposome suspension containing the amount of phospholipids indicated in the Figures.

Analytical procedures

NH_3 was measured by the phenol/hypochlorite method described by Chaney & Marbach [14]. Protein

was determined either by the method of Lowry *et al.* [15], or by the method of Warburg & Christian as described by Layne [16]. Phospholipid phosphorus after mineralization was determined as described by Chen *et al.* [17].

RESULTS

As may be seen from Figs. 1 and 2, maximum velocity of the reaction catalysed by pig heart AMP deaminase was decreased by 50% in the presence of about 15 µM-dioleoylphosphatidic acid both in the presence and in the absence of ATP. Phosphatidic acid exhibited a non-competitive inhibitory effect with a K_i of about 15 µM. Orthophosphate is an effector known to exhibit a negative allosteric effect on AMP deaminase from skeletal muscle, brain [18] and pig heart [8]. In our experiments 10 mM-orthophosphate exerted a 50% inhibitory effect on pig heart AMP deaminase (Fig. 3).

Among various phosphatidates with saturated fatty acyl chains, only dilauroylphosphatidate (DLPA) was found to exert an inhibitory effect on the AMP deaminase activity (Table 1). A 50% inhibitory effect was seen in the presence of 750 µM of this phosphatidate. Dimyristoyl-(DMPA) and dipalmitoylphosphatidate (DPPA) were both without effect on AMP deaminase activity. The transition temperature at pH 6.5 for DMPA ($T_i = 49$ °C), differed markedly from that observed for dimyristoylphosphatidylcholine ($T_i = 23$ °C) as determined by fluorescence of NPN or light scattering. NPN was of limited use for the determination of T_i for the DMPA, which is in accordance with the results of Eibl & Blume [12] who reported these limitations for the ether analogue of natural phosphatidate, 1,2-dihexadecyl-*sn*-glycerol-3-phosphoric acid.

NPN is a fluorescence indicator which distributes between membrane and surrounding medium according to the free volume present in the membrane, and has much higher fluorescence inside the membrane [13]. This indicates some restriction for the penetration of stranger molecules into the bilayer structure of phosphatidate. This restriction has been elegantly overcome by Trauble [11], simply by increasing the pH. Expansion of methylphosphatidic acid bilayers during ionization of phosphate

Fig. 1. The effect of dioleoylphosphatidate containing liposomes on the non-activated AMP deaminase from pig heart

Incubation without liposomes (○), and with liposomes containing phosphatidate at concentrations of: 4 µM (■), 8 µM (□), 16 µM (△) and 20 µM (●). For experimental details see the text.

Fig. 2. Dixon plot presenting the effect of dioleoylphosphatidic acid-containing liposomes on the ATP-activated pig heart AMP deaminase

Incubations were carried out as described in the text at 3 mM- (●), 5 mM- (□) and 10 mM-AMP (○) concentration in the presence of 1 mM-ATP.

Fig. 3. Influence of orthophosphate on the non-activated AMP deaminase from pig heart

Incubations were carried out in succinate buffer. For experimental details see the text.

groups caused by alkalization of the medium, leads to a strong increase in NPN fluorescence due to its increased penetration of the membrane [11]. In our experiments the DLPA membranes underwent gel-liquid crystal transition at about 33 °C in succinate buffer at pH 6.5. Changing pH from the initial 6.5 to 7.9 resulted in a dramatic decrease in T_i from 33 °C to 15 °C (Fig. 4). As may be

seen in Table 1 the DLPA exerted a strong inhibitory effect on the AMP deaminase activity when incubated with the enzyme at pH 7.9. The pH dependence of pig heart AMP deaminase activity revealed that the activity of the enzyme alone at pH 7.9 was little changed compared with that in either succinate buffer or in Mops/KOH buffer at pH 6.5.

DISCUSSION

The acidic phospholipids of cellular and intracellular membranes have been reported to be potential candidates for several functions: (a) activation [19], stabilization [20] and structural organization of the enzymes within the membranes [18], (b) the preferred site of association with cholesterol [21], (c) hormone responsiveness [22], (d) discrimination between permeability of univalent cations [23], (e) electrostatic interactions with proteins [24]. Although they are known to activate protein kinase C [25], the acidic phospholipids seem to act as inhibitors of some of the so-called soluble enzymes [6,9].

The results presented in this paper clearly indicate that the phosphatidate-containing membranes can affect profoundly the activity of heart muscle AMP deaminase (Figs. 1 and 2). Dioleoylphosphatidate bilayers, regarded as a model membrane because they exist in a fluid state under the conditions employed in the absence of divalent cations, exerted the strongest non-competitive interaction with AMP deaminase with a K_i of 15×10^{-6} M (Fig. 2). Orthophosphate, known as a negative allosteric effector of AMP deaminase [8], inhibits the reaction by three orders of magnitude less, the half inhibition being reached at about 10^{-3} M-phosphate (Fig. 3).

This indicates that the immobilizing on the membrane surface greatly potentiates the inhibition exerted by the orthophosphate anion. Intracellular negatively-charged phospholipids may be the reservoirs of divalent cations which condense the membrane [11]. Naturally occurring phospholipids reside intracellularly as a mixture of molecules containing both unsaturated, as well as

Table 1. The effect of phosphatidates containing saturated acyl chains on the ATP-activated pig heart AMP deaminase

AMP and ATP concentrations were 10 mM and 1 mM respectively. Measurements were performed either in 100 mM-potassium succinate, pH 6.5, containing 100 mM-KCl or in 10 mM-Mops/KOH, pH 7.9, containing 100 mM-KCl. Changes in phase-transition temperature (T_i) for each phosphatidate were monitored by following the changes in 90° light scattering at 400 nm of the dispersions containing 5×10^{-4} M lipid in either succinate or Mops/KOH. Transition temperatures (T_i) for phosphatidates investigated in succinate buffer, pH 6.5, were: for DLPA 33 °C; for DMPA 49 °C; and for DPPA 67 °C. The change of pH from 6.5 to 7.9 caused a marked decrease of T_i for DLPA from 33 °C to 15 °C both measurements being performed in Mops/KOH. For other experimental details see the text.

	Succinate buffer, pH 6.5					Mops/KOH buffer, pH 7.9	
	Control	DLPA (375 μ M)	DLPA (750 μ M)	DMPA (750 μ M)	DPPA (750 μ M)	Control	DLPA (750 μ M)
Specific activity (μ mol NH_3 /min per mg of protein)	5.0 (100 %)	3.7 (74 %)	2.5 (50 %)	5.0 (100 %)	5.0 (100 %)	4.4 (100 %)	0.2 (4.5 %)

Fig. 4. The changes of phase transition temperatures of dilauroylphosphatidate membranes dependent on the increasing ionization effect

Two distinct points of the degree of dissociation α : 0.5 and 1.5 (pK_1 and pK_2 respectively) are taken from [12]. Measurements were performed by 90° light scattering at 400 nm for 5×10^{-4} M-lipid dispersion in 10 mM-Mops/KOH buffer containing 100 mM-KCl at the pH indicated. For additional experimental details see text.

saturated fatty acyl chains. Saturated acyl chains containing phosphatidates are either non-inhibitory for the AMP deaminase or inhibit the activity rather poorly (Table 1) compared with unsaturated ones (Figs. 1 and 2). The phase-transition properties of myristic acid-containing phosphatidylcholine and phosphatidate membranes revealed that phosphatidate at the pH employed (pH 6.5) forms a more condensed membrane structure. Control experiments revealed that dimyristoyl-phosphatidylcholine-containing liposomes potentiate the AMP deaminase activity in the presence of ATP in the same way as dioleoylphosphatidylcholine bilayers

do (results not shown). It is of particular interest that the phosphatidate membranes have a strong compact structure despite a negative charge which should expand the surface. The high T_i of phosphatidate membranes may be explained by intermolecular hydrogen bonds whose strength outweighs the destabilizing effect arising from the presence of negatively-charged phosphate groups within the membrane surface [15]. Such stabilized structures form a barrier against the deep penetration of molecules such as NPN and presumably AMP deaminase. This unusual property of phosphatidate bilayer is expected to be observed between two points of dissociation: 0.5 and 1.5 (pK_1 and pK_2). For a degree of dissociation of 2, the stabilization of membranes by interfacial hydrogen bonds is very much reduced. This looser organization of the hydrophobic membrane core might allow NPN molecule to penetrate deeper [12]. Although DLPA displays a rather high T_i (33 °C at pH 6.5), alkalinization of the surrounding medium results in a marked fluidization of the membrane at a constant temperature (Fig. 4). This fluidization allows a stronger interaction between membrane and enzyme molecule (Table 1).

One is tempted to speculate that an effect similar to that shown by the artificial membrane systems may be displayed by the cellular membrane in the heart. Non-inhibited AMP deaminase presumably lowers the concentration of the substrate for 5'-nucleotidase by removing AMP from the adenine nucleotide pool. At the same time the purine nucleotide pool is preserved by providing IMP for the purine nucleotide cycle [26]. Since adenosine, the product of the 5'-nucleotidase reaction, is regarded as an effector in the regulation of the coronary blood flow [27], the reversible association of AMP deaminase with acidic lipid-containing membranes may be of special importance in the regulation of heart function.

This work was supported by a grant from the Polish Academy of Sciences within the project CPBR 3.13.6.1.7.

REFERENCES

- Mitchell, C. D., Mitchell, W. B. & Hanahan, D. J. (1965) Biochim. Biophys. Acta **104**, 348–358

2. Srere, P. A. (1972) in *Energy Metabolism and the Regulation of Metabolic Processes in Mitochondria* (Mehlman, M. A. & Hanson, R. W., eds.), p. 79, Academic Press, New York
3. Duchan, G. & Colier, M. B. (1971) *J. Membr. Biol.* **6**, 138–157
4. Yu, J. & Steck, T. L. (1975) *J. Biol. Chem.* **250**, 9176–9182
5. Karadsheh, N. S. & Uyeda, K. (1977) *J. Biol. Chem.* **252**, 7418–7420
6. Godinot, C. (1973) *Biochemistry* **12**, 4029–4034
7. Godinot, C. & Lardy, H. (1973) *Biochemistry* **12**, 2051–2060
8. Purzycka-Preis, J., Prus, E., Woźniak, M. & Żydowo, M. M. (1978) *Biochem. J.* **175**, 607–612
9. Purzycka-Preis, J. & Żydowo, M. M. (1987) *Int. J. Biochem.* **19**, 565–568
10. Gurr, M. J. & James, A. T. (1971) in *Lipid Biochemistry: An Introduction* (Chapman, D. & Hall, D. A., eds.), pp. 11–36, Academic Press, London
11. Trauble, H. (1976) in *Structure of Biological Membranes* (Abrahamsson, S. & Poscher, I., eds.), pp. 509–550, Plenum Publ. Corp., New York
12. Eibl, H. & Blume, A. (1979) *Biochim. Biophys. Acta* **553**, 476–488
13. Trauble, H. & Overath, P. (1973) *Biochim. Biophys. Acta* **307**, 491–512
14. Chaney, A. L. & Marbach, E. P. (1962) *Clin. Chem.* **8**, 130–132
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
16. Layne, E. (1957) *Methods Enzymol.* **3**, 451–454
17. Chen, P. S., Toribara, T. Y. & Warner, H. (1956) *Anal. Chem.* **28**, 1756–1758
18. Olivecrona, T. & Orelund, L. (1971) *Biochemistry* **10**, 332–340
19. Wheeler, K. P. & Whittam, R. (1970) *Nature (London)* **225**, 449–450
20. Cerletti, P., Giovenco, M. A., Giordano, M. G., Giovenco, S. & Strom, R. (1967) *Biochim. Biophys. Acta* **146**, 380–385
21. Van Dijk, P. W. M. (1979) *Biochim. Biophys. Acta* **555**, 89–101
22. Levey, G. S. (1971) *Biochem. Biophys. Res. Commun.* **43**, 108–113
23. Papahadjopoulos, D. (1971) *Biochim. Biophys. Acta* **241**, 254–263
24. Rand, R. P. (1971) *Biochim. Biophys. Acta* **241**, 823–834
25. Murakami, K., Chan, S. Y. & Routtenberg, A. (1986) *J. Biol. Chem.* **261**, 15424–15429
26. Altschuld, R. A., Gamclin, L. M., Kelley, R. E., Lambert, M. R., Apel, L. E. & Brierley, G. P. (1987) *J. Biol. Chem.* **262**, 13527–13533
27. Berne, R. M. (1964) *Physiol. Rev.* **44**, 1–29

Received 1 March 1988/7 June 1988; accepted 15 June 1988