The Relationship between Cation-Induced Substrate Configuration and Enzymatic Activity of Phosphatidate Phosphohydrolase from Human Liver

Bahram Haghighi*, Mohamad Yari and Shahraz Tori

Dept. of Clinical Biochemistry, Isfahan University of Medical sciences, Isfahan, Iran

ABSTRACT

The mechanism by which bi-and trivalent cations affect human liver phosphatidate phosphohydrolase (PAP) activity was investigated. Bivalent cations up to 1 mM increased PAP activity whereas at higher concentrations the activity of the enzyme decreased. The stimulatory concentration for trivalent cations such as Al^{3+} and Cr^{3+}, however, was much lower being 2 m M and 1 m M, respectively. All cations affecting PAP activity were also able to induce phase transition of phosphatidate from lamellar (La) to inverted hexagonal (H_{II}) form. The rate of La-H_{II} transition was different for each cation. At 100 mM concentration of Mg^{2+} only 26% of the original phosphatidate remained in La form and for other cations tested ranged from 14.5% to 76% The phase transition was blocked by EDTA. Magnesium from 0.8 to 1.5 mM concentration raised PAP activity (3-fold) with La form of substrate but not with the H_{II} phase. Monovalent cations such as Na^{+} and K^{+} neither affected enzyme activity nor substrate configuration. These data suggest that cation-induced PAP activation is not as a result of cation-protein interaction, but is due to formation of a suitable substrate configuration for the enzyme catalysis during phosphatidate phase transition. It

appears that the real substrate configuration for PAP activity is situated between La and H_{II} phases. *Iran. Biomed. J. 4: 13-19, 2000*

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INTRODUCTION

Phosphatidate phosphohydrolase (PAP) (EC.3.1. 34) catalyzes dephosphorylation of phosphatidic acid and is a regulatory enzyme in glycerolipid metabolism [1]. Two different forms of PAP activity are present in animal cells [2, 3]. One form, present in plasma membrane, is insensitive to Mg^{2+} and N-ethylmalemide and is suggested to be involved in signal transduction by modulating the second messengers diacylglycerol and phosphatidic acid [3-5]. Another form of PAP, located in cytosolic and microsomal fractions, is sensitive to N-ethylmalemide and can be activated by Mg^{2+} [4, 6]. This form of PAP translocates between cytosol and microsomes and is considered to be the metabolic form of the enzyme participating in the synthesis of triacylglycerol and phospholipids [7].

Magnesium ion can enhance the enzymatic activity of the purified PAP [8]. The presence of certain detergents, such as Triton X-100 or Tween 20 in the absence of Mg^{2+} also can activated the metabolic form of PAP using membrane bound substrate [8, 9]. Using an aqueous dispersion of phosphatidate, however, reversed the Triton effect [10]. It has been suggested that the detergents alter physical structure of membrane-bound substrate in a way that even in the absence of Mg^{2+} the substrate can properly binds to the enzyme active site where catalytic activity occurs [10]. Butterwith *et al.* [8] have proposed that when Mg^{2+} stimulated the initiation step of the enzyme activity, the removal of the ion by high concentration of EDTA does not affect PAP activity.

The effects of bivalent cations on PAP activity have been a contradictory subject. Primary reports have shown that Ca^{2+} directly inhibits PAP activity [1]. Other investigators have reported that Ca^{2+}

in the absence of Mg^{2+} stimulated the enzyme activity [9]. PAP activity toward membrane-bound substrate in the presence of Mg^{2+} was inhibited by calcium ion [10]. Among other bivalent cations, Zn^{2+} stimulated but Mn^{2+} did not affected PAP activity [8]. In this study, the mechanism by which bivalent cations can affect the PAP activity of human liver and the changes induced by these cations in the substrate configuration were investigated.

MATERIALS AND METHODS

Chemicals. Phosphatidic acid (sodium salt), dithiothreitol, EDTA and imipyramine hydro-chloride were obtained from Sigma Co. (USA). Phenylmethylsulfanylfluoride was from Aldrich Co. (UK). All other chemicals were reagent grade.

Human liver samples. Postmortem liver samples were removed by immediate autopsy after death, caused by accident, in the hospitals of Isfahan University of Medical Sciences. The samples (15-20 g) were washed three times with normal saline and kept the same solution for homogenate preparation.

Homogenate preparation. Each liver sample was perfused with normal saline to remove the remaining blood, cut into small pieces and homogenized in 50 mM Tris-HCl buffer pH 7.4 containing 0.25 M% sucrose, 0.2 mM dithio-threitol, 0.2 mM phenylmethylsulfonyl-fluoride, 1% aprotinine, 0.2 M tetrahydrolipstatine and 0.1 mM $MgCl_2$. None of the proteases or lipase inhibitors affected PAP activity. The homogenate was centrifuged at 10,000 ´ g for 10 min at 4^oC and the supernatant was separated and centrifuged at 12,000 ´ g for 30 min. The upper lipid layer of the supernatant was removed and the lower layer was passed through a column containing glass wool to remove the remaining lipids. This solution, containing cytosolic and microsomal fractions, was kept at -10^oC for determination of PAP activity.

Determination of PAP activity. PAP activity was determined by

measuring inorganic phosphate (Pi) released from an aqueous dispersion of phosphatidate solution [12]. The assay mixture contained 0.1 M Tris-HCl buffer pH 7.4, 1 mM $MgCl_2$ or indicated amount of each cation (see legends), 2 mM phosphatidate, 1 mM dithiothreitol and appropriate amount of the enzyme solution. Substrate and sample blanks were taken in which the enzyme and phosphatidate solutions were omitted, respectively [13]. The assay mixture was incubated for 30min at 37^oC. The reaction was stopped by addition of 1 ml trichloroacetic acid (10%). Hence, the Pi released was measured [14].

The effects of cation on PAP activity. The effect of various cations at different concentrations on PAP activity was measured in 0.1 M Tris-HCl buffer pH 7.4 containing 1 mM dithiothreitol.

Protein determination. Protein was measured by the method of Bradford [15].

Separation of aggregated forms of phosphatidic acid. Aqueous emulsion of phosphatidic acid forms different aggregated structures such as lamellar (La) and inverted hexagonal (H_{II}) phases in the presence of bivalent cations and amphyphilic compounds. Phosphatidate (20 mM) was incubated with each cations (see the results) and after equilibrium the two structures were separated by centrifugation at 1,500 ´ g for 20 min at 4^oC. The supernatant containing La phase was separated and H_{II} phase in the pellet was confirmed by polar solvents (for the presence of salt precipitate) and nonpolar solvents such as Sudan black and Fat red 7 (for H_{II} phase). The precipitate of H_{II} was washed with water and centrifuged for 10 min. at 1,500 ´ g. The absorption increased at 356 nm in 0.1 M Tris-HCl buffer pH 7.4 was taken as the indicator for La to H_{II} phase transition [16].

The effects of cations on La and H_{II} phases. Phosphatidate (20 mM) was incubated in 0.1 M Tris-HCl buffer pH 7.4 in the presence

of each cation (see the results) for 10 min and the change in absorbance at 356 nm was recorded. The La to H_{II} transition was also confirmed by acidic digestion of phosphatidic acid in La phase. The released inorganic phosphate represents phosphatidic acid present in La phase [17]. To the separated La phase 1.5 ml of 70% perchloric acid was added and placed at 180^{o}C for 3 h. The solution was evaporated to dryness and kept at 150^{o}C for 30min . The residue was dissolved in 0.5 ml of H_2O for determination of P*i* released [14].

Preparation of membranes overloaded with phosphatidate. To 5 ml of cytosolic/microsomal fractions of the liver cells 10 to 15ml ether was added and vortexed for 20 min to dissolve the membrane lipids. The ether layer was separated and evaporated to about 2 ml under a stream of nitrogen. To this solution, 5 ml H_2o was added and the remaining ether was evaporated. The remaining solution contained membranes to which phosphatidic acid (100 mM) was added for preparation of overloaded membranes.

RESULTS

The effects of bivalent cations on PAP activity. The activity of PAP was measured in the presence of different concentrations of Mg^{2+}, Ca^{2+}, Zn^{2+}, Cd^{2+} and Ni^{2+} (Fig. 1). These cations activated PAP activity at concentrations of up to 1 mM, after which the activity of the enzyme was declined. Similar patterns were also obtained for Fe^{2+} and Cu^{2+} (data not shown). However, trivalent cations such as Al^{3+} and Cr^{3+}, stimulated PAP activity at 2 mM and 1 mM, respectively (Fig. 2A). No changes in the PAP activity were observed by monovalent cations such as Na^{+} and K^{+} up to 2 mM in the presence or absence of Mg^{2+}.

Lamellar and non-lamellar phases of phosphatidate. All cations

affecting PAP activity (see above) were also able to induce La to H_{II} phase transition of phosphatidate. The data of Mg^{2+}, Ca^{2+} (Fig. 3A), Fe^{2+}, Zn^{2+} (Fig. 3B), and Cr^{3+} (Fig. 2B) all demonstrated a peak of absorbance at cation concentration of around 250-300 mM. Similar results were also obtained with Cu^{2+}, Cd^{2+}, Ni^{2+} Al^{3+} and Mn^{2+} (data not shown). Monovalent cations (Na^{+}, K^{+}) did not affect La to H_{II} transition. The presence of Mg^{2+} together with any other cations caused an additive effect. The major effect of the cations on phosphatidate configuration was obtained about 4 min after incubation, then there after the phase transition completed within 15 min.

The phase transition was also confirmed by digestion of La form remained in the mixture and measurement of the released inorganic phosphate. (Fig. 4) shows that La form decreased as Mg^{2+} or Cd^{2+} concentration was elevated. The same results were observed with other cations mentioned above. The rate of La-H_{II} transition, however, was different for each cation. Only 26% of the original phosphatidate remained in La form at 100 mM Mg^{2+} and for other cations 14.5% to 76% remained. There was no correlation between ionic radius or atomic number and the rate of La-H_{II} transition. Phase transition induced by all bivalent cations
including Mg^{2+}, Ni^{2+} and Mn^{2+} using overloaded membranes showed similar results as those of phosphatidate dispersion (Fig. 5).

Fig. 1. The effects of bivalent cations on human liver PAP activity. The enzyme activity was measured in the presence of indicated concentrations of (A): u , Mg^{2+}; s , Zn^{2+}; n , Ca^{2+} and (B): u , Mg^{2+}; s , Cd^{2+}; n , Ni^{2+} as described in Methods. PAP activity in the presence of 1 mM Mg^{2+} was taken as 100%. Each point represents mean of 2 different experiments.

The effect of chelator on La and H_{II} stability. When in the presence of Mg^{2+} EDTA was added to a dispersion solution of phosphatidate the formation of H_{II} configuration was decreased. EDTA, however, did not affect the phase transition when it was added after H_{II} formation (Fig. 6).

PAP activity with different phosphatidate configuration. PAP activity of cytosolic/ microsomal fraction was measured using endogenous substrate, aqueous dispersion (La phase) and H_{II} phase of phosphatidate in the presence of Mg^{2+} (Fig. 7). The results showed that Mg^{2+} at 0.8-1.5 mM concentration increased PAP activity (3-fold) with La form of the substrate but

Fig. 2. The effect of trivalent cations on human liver PAP activity and phosphatidic acid configuration. (A) The enzyme activity was measured in the presence of indicated concentrations of Al^{3+} (u) and Cr^{3+} (n) as described in Methods. The activity for 1 mM of Mg^{2+} was taken as 100%. (B) The absorbance of phosphatidic acid (20 mM) in 0.1 M Tris-HCl buffer pH 7.4 at 356 nm was measured in presence of the indicated concentrations of Cr^{3+}. Each point represents mean of 3 independent experiments.

has affected slightly PAP activity using either H_{II} phase or endogenous substrate.

DISCUSSION

Several studies have demonstrated the enzymatic characteristics of PAP from various sources [1-2]. The reported results, however, have not provided firm conclusion and to some extent were controversial. Assay conditions and the type of substrate employed are among the

elements by which the enzyme behavior may vary. In this study, substrate configuration and its effect on PAP

Fig. 3. The effects of divalent cations on phosphatidic acid configuration. The absorbance of phosphatidic acid dispersed in 0.1 M Tris-HCl buffer pH 7.4 was measured at 356 nM in the presence of A: Mg^{2+} (u), Ca^{2+} (n) and B: Zn^{2+} (u), Fe^{2+} (n). Each point represents the mean of 3 independent experiments.

activity of human liver was investigated.

It is well established that hydrated natural lipids could form fluid lamellar (La) and non-lamellar phases [16]. The inverted hexagonal (H_{II}) phase is most common non-lamellar phase. The structural properties of the lipids change when the fluid bilayer, La, phase is converted to H_{II} phase. Phosphatidic acid could also form aggregated structures, under different conditions, in which La-H_{II} transition may occur. Hydrophobicity of phosphatidic acid provides a special enzyme-substrate interaction in which the kinetic studies become complicated and differ from those of the classic substrates [18-19].

Although bivalent cations at low concentration stimulate PAP activity at various levels, optimum concentration was the same for all cations tested

Fig. 4. Phosphatidic acid remaining in La form after incubation with cations. Phosphatidic acid (20 mM) was incubated in 0.1M Tris-HCl buffer pH 7.4 in the presence of Mg^{2+} (n) and Cd^{2+} (s), and after equilibrium La phase was separated, digested with perchloric acid and inorganic phosphate released was measured as described in the Method. Each point is the average of 2 independent experiments.

Fig. 5. The effects of cations on the configuration of overloaded membranes. Membranes of human liver cells were overloaded with phosphatidic acid as explained in the Methods. The absorbance of a 20% dispersion of these membranes was measured at 356 nm in 0.1 M Tris-HCl buffer pH 7.4 in the

presence of indicated concentrations of Mg^{2+} (u), Ni^{2+} (n) and Mn^{2+} (s). Each point represents the mean of 3 independent experiments.

Fig. 6. The effect of EDTA on magnesium-induced phase transition of phosphatidic acid. The absorbance of aqueous dispersion of 20 mM phosphatic acid (La form) (n) and equivalent amount of H_{II} phase (s), prepared according to the Methods, was measured in 0.1 M Tris-HCl buffer pH 7.4 containing 0.25 mM MgCl and indicated concentrations of EDTA at 356 nm. Each point represents the average of 2 independent experiments.

Fig. 7. The effect of magnesium and substrate configuration on human liver PAP activity. PAP activity was measured in the assay buffer using endogenous substrate (n) and phosphatidic acid (20 mM) in La (s) and H_{II} (+) forms and in the presence of indicated concentration of Mg^{2+} as described in the Methods. Each point represents the mean of 3 independent experiments.

(Fig. 1 and 2). It appears that the enzyme acti-vation parallels phase transition (La-H_{II}) induced by the cations (Fig. 2 and 3). The enzyme activity, however, declined when La to H_{II} transition was completed. The finding that trivalent cations also mimic bivalent cations in both phase transition and enzyme activation (Fig. 2B), suggested that phase transition (not type of cation) is important in the enzyme activation. Verkleij *et al.* [20] reported that bivalent cations having different chemical properties are able to induce non-bilayer structure in phosphatidic acid containing model membranes.

Therefore, it is likely that cation-induced PAP activation is not resulted from cation-protein interaction but during the phase transition a substrate configuration suitable for the enzyme catalysis is formed. It appears that the real substrate configuration for PAP activity is situated between La and H_{II} phases. The lack of enzyme activity with substrate having only H_{II} configuration suggested that the enzyme can reach the substrate only when the La phase is disrupted and before H_{II}

phase is completely formed (Fig. 7). This configuration exists when the substrate fluidity is elevated. Therefore, cations or amphiphilic drugs that at low concentration stimulate membrane fluidity and at high concentration promote H_{II} formation activate and inhibit PAP activity, respectively [21]. In overloaded membrane during La-H_{II} transition induced by cations (Fig. 5) after La structure is disrupted and before H_{II} phase is formed soluble PAP penetrate the membrane and interacts with the phosphate group of the substrate. When the cation concentration was low in which the equilibrium between La and H_{II} phases took longer time, maximum enzyme activity was observed. At high cation concentration, however, that promotion of H_{II} phase was rapid the enzyme was inhibited.

It has been reported that the stimulatory effects of cations on PAP activity were paralleled with translocation of the enzyme from cytosol to microsomes [22]. Chloropromazine also induces both: enzyme translocation and the phase transition [23]. This compound destabilized the membranes and facilitated the enzyme-microsome interaction. Similar mechanism may be involved in the translocation and activation of PAP from cytosol to the endoplasmic reticulum induced by long chain fatty acids such as oleate [7, 23-25]. In short, the slow phase transition of the substrate and enzyme translocation are both involved in the mechanism by which an activator affects PAP activity. There are other membrane-associated enzymes, similar to PAP, in which the change in membrane structure alters their activities such as Ca-ATPase [9] and mannosyltransferase II [16, 26]. CTP-phospho-cholin cytidyltransferase also obtains its substrate from lipid structure of the membranes. This enzyme translocates between the cytosol and microsomes by long chain fatty acids and fatty alcohols and its maximum activity is obtained during phase transition (La to H_{II}) [27-29].

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