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THE ASSOCIATION OF GLYCOLYTIC ENZYMES WITH CELLULAR AND MODEL MEMBRANES

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Abstract: This article deals with the binding of glycolytic enzymes with membranous or protein subcellular structures. The representative papers of the last three decades dealing with this matter are reviewed. The studies evidencing the binding of some glycolytic enzymes to insoluble subcellular proteins and membranous structures are presented. It is currently generally accepted that the glycolytic enzymes work in some organisation. Such organisation undoubtedly plays a marked role, although still poorly known, in the regulation processes of glycolysis. From this review, the conclusion emerges that the regulatory ability of the binding of glycolytic enzymes to cellular membranes should be added to the list of well-known mechanisms of post-translational regulation of the glycolytic enzymes. Some of the results presented are the background for the hypothesis that planar phospholipid domains in/on the membrane surface are capable of functioning as binding sites for these enzymes. Such binding can modify the conformation state of the enzymes, which results in changes in their kinetic properties; thus, it may function as a regulator of catalytic activity.

Key Words: Glycolytic Enzymes, Membranes, Enzyme-Membrane Interaction, Lipid Bilayers, Lipid-Protein Interaction

INTRODUCTION

This article deals with studies on the association of glycolytic enzymes with the membranous or protein structural components of a cell, as well as with lipid bilayers as model membranes with respect to a possible role of their association in the regulation of glycolysis. All the enzymes of the glycolytic pathway are well soluble proteins and can easily be separated from the insoluble fractions of cells. Classic models of the regulation of the metabolic pathway have been

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evaluated on the basis of the kinetic and molecular properties of the isolated and purified enzymes in highly diluted solutions. Their good solubility and their presence in supernatant fraction after cell lysis were the main background for the concept of their having a cytosolic location, where they can freely diffuse. Using data obtained *in vitro* for a diluted solution to recognize the molecular mechanisms of the activity of the enzymes and subsequently the glycolytic path regulation was justified by the acceptance of the model of cytosol as a reservoir of proteins in aqueous solution. The first reports calling that model into question appeared in the end of the nineteen sixties and in the nineteen seventies, when the ability of some glycolytic enzymes to bind *in vitro* to insoluble fractions of cells [1-7], as well as their ability to complex with each other [8-10] had begun to be documented. If we assume that the organization of the enzymes affects their kinetic properties, this must be taken into consideration as one of the factors in the regulation of the enzyme pathway. In this article, the evidence of the association *in vitro* of glycolytic enzymes with membranous or other subcellular structures as well as with model lipid adsorptive systems are briefly reviewed. Possible mechanisms of the association, related problems and the main conclusions are discussed.

STUDIES EVIDENCING THE BINDING OF GLYCOLYTIC ENZYMES TO SUBCELLULAR STRUCTURES *IN VITRO*

Some of the glycolytic enzymes have been shown, using various techniques, to be able to associate *in vitro* with the insoluble fractions of the fragmented cells of various organisms and various tissues. Below, a brief review of the most representative examples of the studies demonstrating such association is presented. Clarke and Masters demonstrated the adsorption of several glycolytic enzymes (phosphoglycerate kinase, triosephosphate isomerase, phosphoglycerate mutase, enolase and hexokinase) to myofibrillar proteins [7]. These enzymes reveal relatively strong binding, sensitive to ionic strength, to the structural proteins of muscle cells: F-actin and the F-actin-tropomyosin-troponin complex. Concentrations of monovalent metal salts higher than 0.15 M dissociate the binding [7]. Mitochondrial membranes from various tissues were found to be an effective adsorptive system for isozymes of hexokinase [11-16] and lactate dehydrogenase [17, 18]. Isozyme III of hexokinase was localized on the outer side of cell nucleus membrane using immuno-histochemical techniques of labelling with monoclonal antibodies [15]. Phosphofructokinase was evidenced to non-specifically associate with the inner side of erythrocyte

Abbreviations used: ADP - adenosine diphosphate; AMP - adenosine monophosphate; ATP - adenosine triphosphate; FDP - fructose bisphosphate; F-1,6-DP - fructose 1,6-bisphosphate; F-1-MP - fructose 1-monophosphate; G-3-P - glyceraldehyde 3-phosphate; LDH - lactate dehydrogenase; NAD - nicotinamide adenine dinucleotide -oxidized form; NADH - nicotinamide adenine dinucleotide - reduced form; NADPH - nicotinamide adenine dinucleotide phosphate - reduced form.

membranes [19-21]. This enzyme is able to form a complex with muscle filaments [22], calmodulin [23] and with the insoluble fraction of the white muscle of trout [10]. Phosphofructokinase-M from differentiated skeletal C2C12 myotubes and from mouse skeletal muscle extracts was shown to be specifically co-immunoprecipitative with caveolin-3 [24]. Caveolin-3 is a member of the family of integral membrane proteins, which have a crucial role in the transport of lipids and signal transmission [25, 26]. Aldolase and glyceraldehyde-3-phosphate dehydrogenase are the most often studied enzymes in the context of their binding to membranes. Large portions of various isozymes of aldolase have been observed to be bound to precipitated fractions of the cells of nervous tissue [4, 27, 28] and the structural proteins of mammalian muscle cells [2, 3, 29], as well as to erythrocyte membranes [30, 31]. In the case of aldolase, some differences between the association properties have been documented between isozymes type A and C [4, 27]. The association of aldolase with vacuolar H⁺-ATPase E subunits from bovine kidney microsomes and from osteoclast-containing mouse marrow cultures was evidenced by co-immuno-precipitation studies using an anti-E subunit monoclonal antibody [32]. Numerous studies have shown not only the ability of glyceraldehyde-3-phosphate dehydrogenase to bind to membranes *in vitro* [33-35], but also the specific localization of the enzyme on subcellular structure *in situ* [36-38]. Phosphoglycerate kinase is capable of binding to the membrane of human erythrocyte [39]. The *in vitro* association of lactate dehydrogenase with insoluble cell fractions of the rabbit and chicken muscle tissue, brain and liver cell mitochondria was shown to be dependent on pH, ionic strength and the concentration of NADH [17, 18, 40-46]. Some isozyme specificity was observed in this case (LDH); localization near the synaptosome membranes of the nerve cells was observed for the isozymes containing mainly subunits of the M type [44]. The association of the enzyme with rabbit muscle mitochondria was controlled on protein surface charge rather than on the type of the subunits [47]. Isozymes containing both H and M subunits in molecules with basic pI values reveal effective binding at pH = 7. The association of LDH with the internal membranes of mitochondria of cells from many sources was also observed [48-50]. In all the studied cases of lactate dehydrogenase isozymes, their catalytic activity decreased upon association. Most of the glycolytic enzymes (hexokinase, phosphofructokinase, aldolase, G-3-P dehydrogenase, phosphoglycerate kinase, pyruvate kinase and lactate dehydrogenase) were found to be associated with membrane structures in the purified outer segment of rod and cone photoreceptor cells [51,52]. The differences in the intracellular localization of enolase isoforms was demonstrated via immunolabelling and confocal microscopy analyses on mouse striated muscles [53]. The examples reviewed above prove the widespread occurrence of the ability of at least some glycolytic enzymes to reversibly bind to membranous or protein insoluble fractions, depending on the conditions of the medium. However, for some other enzymes of the glycolytic pathway, like glucose-6-phosphate isomerase, triose phosphate isomerase and phosphoglycerate mutase,

no convincing evidence for such a capability has been found. This ability is a property of glycolytic enzymes from various tissues of many species of animal. Organismal or tissue specificity for the enzyme ability does not seem to occur. It seems that it is reasonable to accept, however, real specificity in binding for some isozymes, since a high diversity in the parameters characterizing the binding was documented for isozymes of hexokinase, aldolase and lactate dehydrogenase.

THE BINDING MECHANISMS

In most studied cases, the association of glycolytic enzymes with insoluble cellular fractions has been shown to be sensitive to pH and ionic strength. An increase in ionic strength diminishes the association. These data were the basis of the view that the association is produced by multi-ionic interaction between ionized groups of the enzymes and the molecules of adsorptive systems. However, other kinds of molecular interactions cannot be excluded. In some cases, the sensitivity to ionic bond-modifying conditions is not typical.

For example, for several glycolytic enzymes, the capability of association with isolated myofibrils remains even at a salt concentration of 0.15 mol/dm³ [7]. The binding of phosphofructokinase-M to caveolin-3 is only disrupted by high ionic strength (>500 mM NaCl) but this association is highly regulated by extracellular glucose and those intracellular metabolites which are allosteric effectors of phosphofructokinase [24]. Caveolins are a family of widely-distributed proteins in cell plasma membranes; they function as scaffolding proteins to locally concentrate many proteins involved in signal transmission in cells. The caveolar organization of signaling proteins and certain glycolytic enzymes could help to couple the generation of cellular energy with environmental cues provided by extracellular factors (such as concentration of glucose and receptor ligands) [24]. The association of aldolase with F-actin is inhibited by increasing salt concentration, but the nature of the inhibition depends on the type of ions [3]. Potassium phosphate is a more effective salt compared to potassium chloride or acetate, but while it is very effective in the case of aldolase, it is almost ineffective for the association of glyceraldehyde-3-phosphate dehydrogenase [3]. The contribution of hydrogen bonds in the association is very likely. The interaction of the hydrophobic fragment of the enzymes with the hydrocarbon core of the lipid bilayer in membrane structures should be also taken into account. The binding of isozyme type I of hexokinase to external mitochondrial membranes strongly depends on the hydrophobic N-terminal fragment, which penetrates the membrane hydrocarbon core [54, 55]. The hexokinase isozyme of type II, in which the fragment is less hydrophobic, reveals a lower affinity to the membranes [13]. Moreover, the dissociating action for lactate dehydrogenase binding to the membranes of such specific agents as NADH, ATP, etc. at low concentrations does not correlate well with the simple model of ionic nonspecific binding [17, 56]. The binding sites on membranes for

the enzymes theoretically could be of two types of nature: 1) binding sites on integral membrane proteins; and 2) membrane binding sites formed by lipid domains. Specific strong binding site(s) on membrane proteins have been found in a few cases. An example of such a protein is band 3 protein in the cytoplasmic membrane of human and mammalian erythrocytes [30, 57, 58]. It is an integral membrane protein responsible for anion transport [58]. The binding site for several glycolytic enzymes is formed by the cytoplasmic fragment of the polypeptide chain, which is rich in carboxyl groups providing a negatively charged domain. Proteolysis or the chemical modification of this fragment diminishes the association.

LIPID BILAYERS AS AN ADSORPTIVE SYSTEM

The assumption of a domain structure of the membranes allows us to postulate that relatively stable lipid domains could be effective binding sites for the enzyme. The occurrence of anionic phospholipid domains not covered by proteins provide charged surfaces of various size. In the studies where model lipid membranes (liposomes, monolayers, "black" lipid membranes) were used, it was shown that the surface of the anionic phospholipid bilayers can be an effective adsorber for many glycolytic enzymes analogously to natural membranes. The observed differences in the association characteristics of various enzymes are explainable by the assumption of the specificity of the binding sites on the molecules of the enzymes. Fructose-diphosphate aldolase efficiently binds to anionic phospholipid liposomes as well as to a planar lipid bilayer formed of the erythrocyte membrane lipids [59-62]. This binding is specific for inositol phospholipids and it is competitive with the enzyme substrate. Glyceraldehyde-3-phosphate dehydrogenase can also be associated with monolayers and liposomes made of the erythrocyte membrane phospholipid fraction or purified anionic phospholipids [63-67]. Liposomes made of the lipid fraction of bovine erythrocytes are able to associate with lactate dehydrogenases from bovine skeletal and heart muscles dependently on pH and ionic strength, but also on the presence of NAD and NADH [56, 68, 69,]. More specific interaction, but with liposomes made of phosphatidylserines, was shown in our laboratory for pyruvate kinase from bovine heart muscle [70]. As mentioned above, in some cases, it has been shown that the association is sensitive to the presence of the enzyme substrates, products and/or specific cofactors at concentration values similar to those of the enzymes. For example, the binding of aldolase to muscle F-actin as well as to membrane fraction rat brain cell homogenate is strongly inhibited in the presence of the substrates (F-1,6-DP, F-1-MP) and of AMP, ADP and ATP [3, 4]. A similar, dissociative influence of such substances can be observed in the case of phospholipid bilayers. A simple model of multielectrostatic adsorption where only the net charge of the membrane surface and the enzyme molecule is considered is not sufficient here. The observed effects become explainable if one assumes the existence of a

conformation-dependent binding site (domain) in the enzyme molecule. The spatial distribution of charged groups is probably crucial for the formation of the site(s). The binding of metabolite molecule(s) probably allosterically modifies this configuration. Based on the evidence of functionally specific domains which have been already recognized for other groups of membrane-related proteins, the occurrence of specific structural domains in the enzymes responsible for complexation with specific membrane components may be postulated. Phosphoinositide (PI)-binding, NADPH oxidase homology domains (PX) target a variety of cell-signaling proteins to organelle membranes through the interaction between the PX domain and specific phosphoinositides [71]. A crucial role in the membrane localization of many proteins involved in cellular signaling, cytoskeletal organisation and lipid transport has also been recently evidenced for the pleckstrin homology (PH) domains [72]. The PH domains were first identified in pleckstrin – the protein kinase C substrate in platelets [73]. The domains appear to be highly conservative in their three-dimensional organisation, and interact directly with the cell membrane by very specific binding to phosphoinositides and, to a much lesser extent, to other kinds of phospholipids (e.g. phosphatidylserines). We are not aware of any studies investigating the three-dimensional structures of the glycolytic enzymes to look for similar domains in their molecules. Speculating on the case of glycolytic enzymes: if they, or some of them at least, had functionally similar modules in their structure, they would probably be good ligands for the phospholipid binding sites in membranes. In addition, it was shown for several proteins that contain PH domains that they are involved in protein-protein complexes, e.g. they bind to filamentous actin and induce actin bundle formation [72]. A recent report dealt with the direct interaction of aldolase C and aldolase A with phospholipase D, a protein involved in many membrane-dependent processes, such as signal transduction pathways, glucose transport, etc., via its PH domain [74]. Aldolase A was found to interact with the PH domain specifically, but not with other PH domains. This would suggest that the PH domain can provide a binding site for aldolase, but again, the specificity is created by the structure of the adsorbed enzyme. So we are of the view that some membrane or protein cellular structures provide rather non-specific adsorptive sites for the enzymes. The individual properties of the enzymes are probably responsible for the observed specificity. According to this view, we believe that the charged surface of the membrane phospholipid domain can generate similar interactions, as well as binding sites for the enzymes. In numerous *in vitro* studies, activity-modified binding of aldolase, glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase and pyruvate kinase to acidic phospholipid bilayers has been proved. It was evidenced that the alteration of lactate dehydrogenase activity and its tryptophanyl fluorescence needs the bilayer structure of phospholipids [75, 76]. Similarly to the interaction with natural membrane preparations, the interaction with lipid bilayers is sensitive to agents modifying ionic bonds, and results in the alteration of the kinetic properties of the enzyme.

MODIFICATION OF KINETIC PROPERTIES OF THE ENZYMES UPON THE ASSOCIATION

The association of the enzymes with membranes, lipid structures and other proteins results in changes in their catalytic activity and other kinetic properties. Below, a few examples of the studies documenting this are presented. The association of aldolase with the F-actin-tropomyosin-troponin complex alters the dependence of the reaction rate on substrate concentration and increasing V_{\max} and K_m [4]. Lactate dehydrogenase isolated from rabbit liver mitochondria has a specific activity 20% lower when it is adsorbed on mitochondrial membranes [18]. Changes in the kinetic mechanisms and parameters of substrate inhibition were also documented for the association of LDH isozymes with chicken liver mitochondria [17]. The binding of the lactate dehydrogenase isozyme A_4 to the structural proteins of muscle cells lowers its V_{\max} and makes the enzyme resistant to substrate inhibition [35]. It is noteworthy that the enzymes are highly differentiated in terms of the modification of their kinetic properties upon adsorption. LDH isozyme A_4 efficiently binds with subcellular structures with the modification of its kinetics, while LDH- B_4 is lacking in this capability.

A profound analysis of the differences in the structure and kinetics between bacterial and vertebrate LDHs led Marmillot *et al.* [77] to the suggestion that the intrinsic allosteric properties of bacterial enzymes were lost through evolution. These properties were replaced in vertebrates by a protein-protein-dependent (LDH-tubulin interaction) allosteric regulation. This ability is probably related to the presence of the N-terminal 15 amino acid fragment in the molecules of vertebrate LDHs. Thus, the alteration of these properties of the glycolytic enzymes occurring *in vivo* would be one of the factors of post-translational regulation of these enzymes, and it should be considered in the evaluation of new models of the regulation of the pathway.

CHANGES IN THE CONFORMATION OF THE ENZYMES

Theoretically, there are several possible mechanisms of the modification of the activity and kinetic properties of the enzymes induced by the type of the interactions:

- as a result of changes in the local concentration of such factors of the reaction as H^+ , salts, substrates and coenzymes, which are produced by changes in the charged group configuration and hydration around the enzyme molecule,
- through steric hindrance for substrates and cofactors to catalytic sites on the enzyme molecule adsorbed on the membrane
- through changes in the enzyme conformation that may also result in changes in the properties already mentioned above or in the affinity of the substrate and cofactors to the enzyme. A combination of these mechanisms may take place as well.

There is little information on the conformational effects in glycolytic enzyme molecules induced by their association with membranes or adsorptive proteins.

Conclusions that such effects occur can be drawn indirectly from the influence of the substances which are specific for the particular enzyme. For example, the effect of the substrate analogs for aldolase [78, 79], namely the coenzyme and its analogs in case of dehydrogenases [56, 68, 80], on the enzyme adsorption on natural as well as artificial lipid membranes strongly suggests conformational mechanisms. A very specific effect of the various intracellular metabolites on the interaction of phosphofructokinase with caveolin-3, mentioned above, was observed. The interaction is highly regulated by the concentration of glucose, and is sensitive to the relevant intracellular metabolites, such as fructose 1,6-bisphosphate and fructose 2,6-bisphosphate, which are allosteric activators of the enzyme [24]. This conclusion of the importance of the conformation in the association has been supported by the results of more direct methods. Techniques of fluorescence spectroscopy are particularly useful in studies of these mechanisms. In our laboratory, we evidenced the conformational rearrangement induced in FDP aldolase, glyceraldehyde-3-phosphate dehydrogenase by the association with phospholipid bilayers [61, 81, 82]. The interaction with phosphatidylinositol liposomes markedly decreases the aldolase tryptophanyl fluorescence and shifts the maximum wavelength toward the "red". The quenching constant value for the aldolase native fluorescence quenching by dynamic quencher (acrylamide) is higher than that for the unbound enzyme. This means an increase in the accessibility of the tryptophanyl chromophores to the small polar molecules of the probe. Unmodified and liposome-interaction-induced conformational states of aldolase reveal a different temperature dependence of the tryptophan residue exposure [61]. In the case of glyceraldehyde-3-phosphate dehydrogenase, external covalently-bound fluorescence probes were used to monitor possible conformational modifications of the enzyme molecules. The probe was formed during the reaction of o-phthaldialdehyde with some amino groups of the protein. This probe was an acceptor of the excitation energy transferred from the native tryptophanyl residues of the protein. Conformational changes were concluded on from the investigation of the observed decrease of the energy transfer efficiency upon binding with phosphatidylinositol liposomes [81, 82].

CONCLUDING REMARKS

In spite of the large amount of data collected, including those on intracellular localization obtained from the studies *in situ*, the problem of whether the association of the glycolytic enzymes occurs *in vivo*, has not been solved yet. The view that the enzymes form complexes and/or are associated with subcellular structures arose mainly from indirect investigation *in vitro*. As the procedures of such investigation produce some perturbation in the studied object and since we do not know about the local conditions in compartmentalized cells, the conclusions coming from the studies bear uncertainty if related to intact cells. However, all the studies performed *in vitro* have provided evidence that an

association regulated by environmental conditions is possible. The growing amount of data on the intracellular localization of the enzymes obtained from the studies *in situ* supports the thesis. Since the binding can alter the kinetic properties of the enzymes, it would be a factor of great importance for the regulation of glycolysis. Reversible binding would be a regulating factor switching the different properties of bound and unbound enzymes. Further development of the techniques visualizing processes in living cells and getting more detailed data on the intracellular local conditions will be crucial for solving these problems.

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