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INTERACTION OF YEAST 3-PHOSPHOGLYCERATE KINASE WITH NEGATIVELY CHARGED CARRIERS

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The aim of this study was to investigate the possibility of an interaction of yeast 3-phosphoglycerate kinase with negatively charged carriers such as polyanionic agents or a polarized electrode. Various polyanions were found to promote enzyme aggregation as judged by ultracentrifugation measurements and chemical modification. The data obtained suggest that these interactions are mediated through the N-terminal domain of the protein. However, the most striking property of 3-phosphoglycerate kinase described here is concerned with its significant dipolar moment as evidenced by electrocapillary measurements, which allows an orientation of the macromolecule in an electric field. Further, the enzyme could be adsorbed by a negatively charged surface, first by hydrophobic links and then oriented perpendicularly to the surface. Therefore, the intrinsic properties of yeast 3-phosphoglycerate kinase agree with the formation of an enzyme-membrane complex and afford the ability for a specific orientation of the molecule at the lipid bilayer surface or in the cytoplasm.

1. Introduction

As early as 1960, Green et al. [1] had suggested that the glycolytic enzymes, which until now had been considered as cytoplasmic enzymes, might be associated to membranes in red blood cells and yeast cells. Thus, as repeating units, they would form a multienzyme complex. Later on, Proverbio and Hoffman [2] presented a hypothesis that ATP generated by membrane-bound 3-phosphoglycerate kinase is compartmentalized in the membrane and utilized by $(Na^+ + K^+)$-ATPase. However, this concept is not well established and the precise role of such well documented associations is always open to discussion.

Glycolytic enzymes in some cases can form cytoplasmic multienzyme particles [3]. They can also interact with structural proteins such as actomyosin in muscle cells [4]. Therefore, these enzymes could be associated with various cellular components.

The association of glyceraldehyde-phosphate dehydrogenase and 3-phosphoglycerate kinase with membranes or other material has often been described [5] and some recent studies have established the parameters of this association. Thus, De and Kirtley [6] gave evidence of a strong and reversible association of 3-phosphoglycerate kinase with erythrocyte membranes. Their results also suggest that 3-phosphoglycerate kinase could interact ionically with the membrane.

To test such types of interaction, we have studied in the present paper the nature of the binding of yeast 3-phosphoglycerate kinase with polyanionic compounds and with a polarized electrode chosen as a model of an interface with a controlled value of the superficial charge.

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2. Material and methods

3-Phosphoglycerate kinase from yeast was prepared according to Scopes [7]. Its specific activity was about 1300 units at 30°C and pH 7.5 [8].

Poly(glutamic acid) (mol. wt. 70000), poly(vinyl sulfate), chondroitin sulfate and polyphosphate (P31) were obtained from Sigma. Heparin was provided by Prolabo; tripolyphosphate was obtained from Billault and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) was purchased from Pierce Chemical Co. All other chemical reagents used were of analytical grade.

Protein concentration. The concentration of 3-phosphoglycerate kinase was derived from the absorbance at 280 nm using $A^{1\%}_{1cm} = 5.0$ [9].

The molar concentration was determined using a molecular weight of 45000 [10].

Reaction with DTNB. 3-Phosphoglycerate kinase (0.8 mg/ml) with or without ligands in 0.02 M Tris-HCl, pH 8, was incubated at 30°C in a spectrophotometric cell. The reaction was started with 1 mM DTNB. The rate of modification of cysteine is related to thionitrophenolate and monitored at 412 nm [11].

Difference spectra. Difference spectra were obtained with a Cary Model 219 spectrophotometer using matching pairs of 0.437 cm light-path cells as previously described [12,13].

Ultracentrifugation experiments. An MSE analytical ultracentrifuge, supplied with a monochromator and a photoelectric scanner, was used. Samples were analyzed in a six-hole rotor using cells of 1 cm light path. Sedimentation velocity was determined from the absorbance at 278 nm recorded versus r.

The measurements of $\log r$ versus time were fitted by regression analysis using an Olivetti P 6060 computer.

Electrocapillary measurements. The variation of the interfacial tension with the potential applied to a dropping mercury electrode with respect to a reference electrode was determined using an automatic drop time recording technique [14]. The capillary was standardized with respect to a solution whose interfacial tension at varying potentials is known, 0.1 M KCl in the present case [15]. 0.1 M KCl was used as supporting electrolyte.

The results obtained being independent of the mean drop time (3–15 s), the adsorption can be considered as instantaneous.

3. Results

The loss in the reactivity of DTNB towards the single cysteine residue of yeast 3-phosphoglycerate kinase [16] constitutes evidence for the interaction of ligands with the anionic site of the enzyme [10,17].

Using this method, the possible interaction of various biological and synthetic polyanions carrying carboxyl (poly(glutamic acid)), phosphate (tripolyphosphate, P31) and sulfate groups (heparin, chondroitin sulfate and poly(vinyl sulfate)) with the enzyme has been tested. These compounds are very different but all possess, in common, negatively charged groups, and afford a total protection against DTNB reaction. Furthermore, by varying the carrier concentration at a fixed enzyme concentration, we can determine in each case the residual free enzyme by its reaction with DTNB

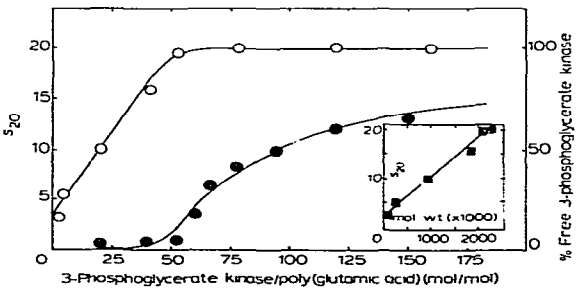

Fig. 1. Stoichiometry of 3-phosphoglycerate kinase-poly(glutamic acid) interaction determined by analytical ultracentrifugation and protection against DTNB reaction: 20 μM enzyme in 10 mM Tris-HCl, pH 8.0. (○) s_{20} and (●) percent free 3-phosphoglycerate kinase (determined by its reaction with 1.0 mM DTNB) are plotted versus various 3-phosphoglycerate kinase/poly(glutamic acid) ratios (mol/mol); (————) theoretical curves assuming a stoichiometry of 48 mol/mol and a dissociation constant $K_d = 1 \times 10^{-7}$ M. Inset: s_{20} plotted versus mean molecular weight determined from percent free 3-phosphoglycerate kinase and therefore number of moles of bound 3-phosphoglycerate kinase/mol poly(glutamic acid) (curve ●).

Table 1

Characteristics of yeast-3-phosphoglycerate kinase aggregation with various polyanions

Polyanion	Mean molecular weight	100% protection effect against DTNB reaction obtained for an enzyme/anion ratio of		Maximum s_{20} obtained for an enzyme/anion ratio of			Mean molecular weight of aggregate (× 1000)
		(w/w)	(mol/mol)	(s_{20}, S)	(w/w)	(mol/mol)	
Carboxylate							
Poly(glutamic acid)	70000	31	48	20	31	48	2230
Phosphate							
Polyphosphate (P31)	3200	14	1	3.2	14	1	48
Sulfate							
Poly(vinyl sulfate)	25000	20	11	30	23	13	520–610
Chondroitin sulfate	40000	27	24	15	27	24	1120

and thus ascertain the amount of enzyme bound to polyanions.

Fig. 1 shows the results obtained with poly(glutamic acid). The experimental data can be fitted by calculated curves assuming, to a first approximation, equivalent and independent binding sites with a dissociation constant $K_d \leq 1 \times 10^{-7}$ M. Then calculations are made using the classical equation $\bar{v} = nk[A]/1 + k[A]$, where $\bar{v}$ is the total average number of occupied sites per molecule of polyanion, n the total number of sites, [A] the concentration of free ligand, i.e., 3-phosphoglycerate kinase and k the association constant.

In fact, the enzyme concentrations used in these experiments (fig. 1) are at least two orders of magnitude higher than K_d and thus neither a possible cooperativity nor a K_d value can be estimated.

Table 1 summarizes the stoichiometry of these aggregations. We observe that only 1 mol of 3-phosphoglycerate kinase is bound to polyphosphate P31 while aggregates were formed with poly(glutamic acid), chondroitin sulfate and poly(vinyl sulfate).

The interaction of the enzyme with tripolyphosphate, the smallest polyanion tested, is strong. Its K_d estimated from the protection effect towards SH-group modification is about 3 μM. The association of other compounds with the enzyme is still stronger.

Furthermore, as shown for poly(glutamic acid), the interaction is dependent (fig. 2) on the ionic strength, a value of 0.3 being sufficient to abolish any association.

3.1. Ultraviolet difference spectrophotometry

The association of poly(glutamic acid) with yeast 3-phosphoglycerate kinase induces the small spec-

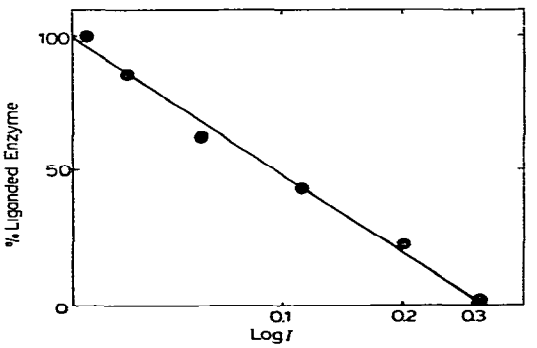

Fig. 2. Ionic strength effect on the stability of the 3-phosphoglycerate kinase-poly(glutamic acid) complex. Percent of the liganded enzyme is determined from the protective effect against DTNB reaction (experimental conditions of fig. 1) with a 3-phosphoglycerate kinase/polyanion ratio of 45, at the indicated ionic strength (ionic strength fixed with KCl).

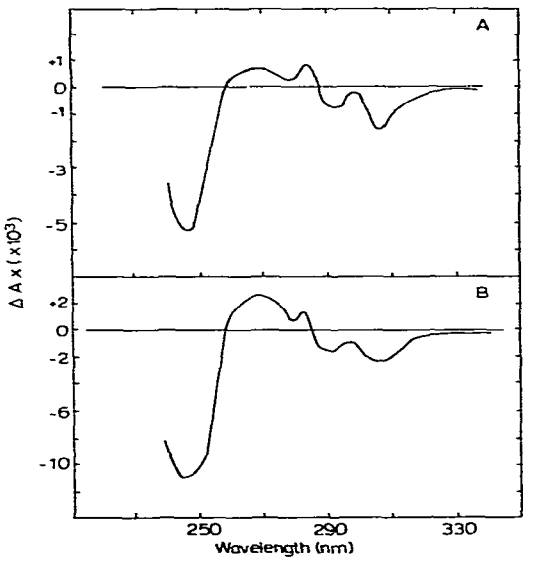

Fig. 3. Difference spectra induced by poly(glutamic acid) binding to 3-phosphoglycerate kinase. Comparison with the effect of sulfate binding. Temperature 19°C. 0.437 cm light-path cells. (A) 38 μM enzyme and 3 μM poly(glutamic acid) in 0.05 M Tris-HCl, pH 7.5. (B) 33 μM enzyme and 10 mM sulfate in 0.02 M Tris-HCl, pH 7.5.

tral effect shown in fig. 3 and is characterized by a major minimum at about 245 nm and minor peaks in the aromatic amino acid region (fig. 3). The estimated value of the maximum magnitude at 245 nm is 270 M^{-1} cm^{-1}.

Similar characteristic spectra are also obtained with the other polyanions.

3.2. Ultracentrifugation

The aggregate formation was followed by analytical ultracentrifugation. The sedimentation coefficient was determined for complexes formed at various enzyme/carrier ratios. These data also lead to quantification of the stoichiometry of the various complexes. Fig. 1 shows the results obtained for poly(glutamic acid).

In these experiments, the enzyme only presents an absorption at 278 nm and free poly(glutamic acid) cannot be detected. Thus, we observe a single peak of sedimentation related to aggregates. However, at low poly(glutamic acid)/3-phosphoglycerate kinase ratios an additional peak with a sedimentation constant s_{20} of about 3 S is observed. The last peak corresponds to free enzyme. The apparent sedimentation constant s_{20} is determined from the top of peaks related to the derivative of the absorbance at 278 nm along the centrifuge cell. This constant varies within experimental precision, proportionally with the size of the aggregate (fig. 1). The stoichiometry of this association is 48 mol 3-phosphoglycerate kinase/mol poly(glutamic acid). All other results which are in agreement with those derived from DTNB reaction are presented in table 1.

3.3. Adsorption

Utilizing the relationship, $\Gamma = -(\partial\gamma/\partial\mu)_E$, where μ is the chemical potential of the uncharged substance and γ the interfacial tension, the relative surface excesses for any value of the electric potential E of the electrode have been calculated for solutions at 2.16, 4.43, 8.25, 12.0, 14.66 and 17.7 μM.

The linear shape of the curves, $\gamma = f(\log C)$, for different values of E shows that the relative surface excess is independent of the concentration and that a saturation of the adsorbed layer is reached for all the concentrations studied, which reveals the strong adsorption of the enzyme.

The Lippmann equation $\sigma^M = -(\partial\gamma/\partial E)_\mu$ gives the variation of the charge of the electrode σ^M with E which in turn enables one to deduce the variation of Γ with σ^M, as shown in fig. 4, from which several results can be deduced.

(1) The general shape of the curve corresponds to the adsorption of an uncharged substance [18] with desorption at high negative charges, which confirms the neutral character of the enzyme.

(2) The region of charge of maximum adsorption (-8 to -10 μC cm^{-2}) is more negative that that corresponding to the adsorption of small neutral molecules such as alcohols [18] and amides [19,20]. The dipole moments of these two types of compounds are, respectively, 2 and 4 debye [21], while their point of maximum adsorption corresponds to charges of -2.5 and -5 μC cm^{-2}. An

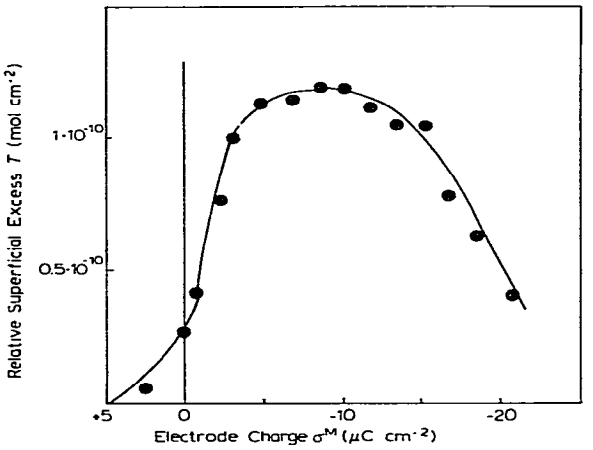

Fig. 4. Variation of the relative surface excess Γ with the electrode charge σ^M.

extrapolation of these results suggests that the enzyme possesses a strong dipole moment of about 10 debye.

(3) The enzyme is practically not adsorbed for positive charges of the electrode as in the case of positively charged surfactants [22]. When $\sigma^M = 0$, i.e., in the absence of any electrical field, $\Gamma = 0.25 \pm 0.05 \times 10^{-10}$ mol cm^{-2}; which implies that each enzyme molecule occupies an area at the surface of the electrode of between 600 and 800 Å^2. This would be compatible with an adsorption of enzyme perpendicular to the electrode in a compact monolayer as the lying-down position would correspond to a much greater area, 2000 Å^2, which in turn corresponds to $\Gamma \approx 0.09 \times 10^{-10}$ mol cm^{-2}.

(4) The significant increase in Γ when the charge becomes negative indicates the formation of multilayers when the electric field is highly negative.

4. Discussion

3-Phosphoglycerate kinase associated to membranes would be located in a polyanionic microenvironment created by negatively charged lipids and intrinsic proteins and submitted to an electrostatic potential [23]. On the other hand, the association to the membrane must require some intrinsic specific properties related to the molecular three-dimensional structure of the enzyme itself. Taking into account these data, we have tested the behavior of 3-phosphoglycerate kinase in model media similar to the membrane environment.

The X-ray data show that yeast and muscle 3-phosphoglycerate kinases are asymmetric molecules organized in two distinct domains of almost equal size separated by a narrow waist [24,25]. Their overall dimensions determined from crystallographic data are $35 \times 45 \times 85$ Å [26]. Hence, the molecule could be assimilated to three spheres of radii 18, 10 and 20 Å, respectively [26]. One domain which corresponds to the C-terminal part of the polypeptide chain includes the nucleotide-binding site [27], the other probably contains the 3-phosphoglycerate-binding site near arginine residues [25], the single cysteine residue, and is characterized by a high level of arginine residues [28]. Although the amount of amide groups is not well known, it seems that a dissymmetric distribution of the charged residues occurs along the two domains, the N-terminal lobe possessing more positive charges than the other [25,27,28]. However, the enzyme is almost a neutral protein. Its pK_i is about 7.2 [16]. These data are in agreement with the high value of the dipolar moment deduced from electrocapillary measurements.

This constitutes a new and important property of the enzyme which would confer a possible orientation of the enzyme by electric fields created by the lipid bilayer or other macromolecules in the cell.

Wrobel and Stinson [17] presented evidence for an anionic site in yeast 3-phosphoglycerate kinase distinct from the active site. This secondary site possesses a high affinity for various anions, in particular for sulfate [29], and would probably be related to the second substrate site described by Schierbeck and Larsson-Raznikiewiecz [30]. In a previous paper [10], we have shown this anionic site to be different from the 3-phosphoglycerate site.

Further, the interaction of anions induces a change in the microenvironment of the cysteine residue located in the same domain which is then

not able to react with DTNB. This property allowed us first to evidence the formation of high aggregates with poly(glutamic acid), chondroitin sulfate or poly(vinyl sulfate), and second, to ascertain the localization of the contact area between the enzyme and polymers in the N-terminal domain.

The binding of 3-phosphoglycerate kinase with high molecular weight polymers as well as with tripolyphosphate and sulfate [10] gives rise to similar spectral patterns. Therefore, all these ligands would be attracted by the same area. The ionic nature of this association is underlined by the dissociation observed at high ionic strength. It is interesting to note that De and Kirtley [6] presented an identical pattern of dissociation of the 3-phosphoglycerate kinase/erythrocyte ghost membrane with a 50% effect at about 0.09 M NaCl, as observed in the poly(glutamic acid)-enzyme aggregate (fig. 2). Such an ionic strength is compatible with an in vivo process.

Furthermore, the nature of the matrix bearing the negatively charged groups does not seem an important parameter. In fact, the number of moles of 3-phosphoglycerate kinase bound seems to be related to the length rather than to the number or the nature of the anionic groups of the polymer. In particular, the binding of 1 mol of enzyme requires about 8 mol of glutamic acid in the chain. The bulkiness of these charged groups along the chain is in agreement with the size of one domain of the enzyme.

The results obtained at an electrode for zero or weakly negative charges show, from the area of occupation of each molecule, that the surface would be saturated with the enzyme oriented perpendicular to the surface. This strong adsorbability of the macromolecule must be associated with hydrophobicity [31], while the influence of the electric field must be related to its high dipolar moment, allowing orientation of the enzyme. Since only one of the two lobes of the enzyme can be adsorbed at negatives charges, it must be that which is charged positively. The formation of multilayers for charges equal to or more negative than 1 μC cm^{-2} must be due to the fact that the electrical field resulting from a charged electrode must extend beyond the first adsorbed layer. Once this is formed the enzyme molecule can still form additional layer, under the influence of the remaining weaker field. This phenomenon can be repeated, the molecules being orientated as in the case of the first layer, provided the electrical field has not been completely neutralized. This explains the increase in the value of the relative surface excess up to the point of desorption due to the greater repulsive force acting on the molecular dipole as in the case of smaller molecules [18].

The choice of the capillary electrode technique as a membrane model is dictated by the following considerations. Several studies have shown that in the case of adsorption at electrodes it is possible to simulate the action of electrostatic and hydrophobic forces under other conditions [32–34]. Furthermore, it has also been shown that the classical electrochemistry of the double layer appropriate to materials which operate as electrodes constitutes a limiting description for different nonelectrode material including colloïds and biosurfaces [35]. The similarity is all the more striking that the specific reaction forces between the adsorbed and adsorbing substances are weak which is true of numerous different compounds (R. Bennes, D. Schuhmann and P. Vanel, unpublished data) but not the case when these contain some very reactive groups [36]. At this stage it may be relevant to discuss the results described above concerning the interaction of the enzyme with either a polyion or an electrode in the light of what is known in the case of ionic micelles and biosurfaces, including lipid bilayer membranes, the behavior of the polyions also being largely dependent, as in the case mentioned above, on the competition between hydrophobic and electrostatic forces [37,38]. The molecular area of the polar head in the case of lipid bilayer membranes has been evaluated as 50 Å^2 [38]. Such a value would correspond to a surface charge of -30 μC cm^{-2} if all the lipids were ionized. As one generally considers that this is only true in the case of 20–40% of the molecules [38], the charge would at the least be -6 μC cm^{-2}. Beyond this, however, one must also envisage the neutralization of part of the dissociated lipids by the cations of the electrolyte in solution. For alkyl sulfates, sodium or alkyl benzenesulfonates, a 60–80% neutralization is found

[39,40]. This leads to a further reduction in the value of the effective charge at the surface of the lipid bilayer membrane and one reaches a minimum value of $-1\ \mu C\ cm^{-2}$, while that of the proteins, at the surface of the membrane, when such substances are present, is probably greater. Now for a charge of $-1\ \mu C\ cm^{-2}$ and an ionic strength of 0.1 M similar to that existing around the membrane, in the case of an electrode the adsorbed proteins already form a bilayer on the surface. It appears therefore that in the case of an enzyme which is globally neutral but the carrier of a highly positively charged group, this substance can be strongly bound through its positive lobe while the negative half of the compound molecule is directed towards the solution.

The measurements of the interfacial tension with capillaries having differing drop times between 1 and 30 s have shown that even in the case of those with the shortest dropping rates, equilibrium was attained. This observation is in agreement with other observations according to which the formation of membrane-protein links is a rapid process taking place in less than 1 s [41].

There remains the question as to the biochemical significance of the formation of superposed layers of proteins on a negatively charged electrode. As under normal conditions the enzyme does not tend to crystallize in solution, one would be tempted to suggest that this could be possibly induced by a high localized electric field. Thus, even in this absence of a surface leading to a layer structure as in the case of an electrode, the influence of a high electric field could lead to interactions between the positive and negative lobes of a macromolecule and the formation of an elementary lattice constituted of two macromolecules as revealed by crystallographic studies [42].

In conclusion, the intrinsic property of 3-phosphoglycerate kinase must make it possible for this enzyme to be absorbed in vivo not only at the membrane but also at other macromolecules such as actomyosin filaments [4] or other glycolytic enzymes [3]. Our results show that the associations are first mediated by hydrophobic links through the N-terminal domain. Second, ionic interactions are also partly responsible for these associations, the orientation being induced by the high dipole moment.

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