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INTERACTION BETWEEN D-GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE AND 3-PHOSPHOGLYCERATE KINASE LABELED BY FLUORESCEIN-5'-ISOTHIOCYANATE: EVIDENCE THAT THE DYE PARTICIPATES IN THE INTERACTION

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SUMMARY: An interaction of rabbit muscle D-glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase labeled with FITC was studied by following the changes in fluorescence intensity of the bound dye. The association between the two enzymes was found to be a rather slow process characterized by a second order rate constant of $1.1\pm0.2\cdot10^3$ $M^{-1}s^{-1}$, the K_D of the complex between apoenzymes being $3.2\cdot10^{-7}$M. The stability of the complex increased upon increase of temperature and ionic strength of the medium, suggesting a hydrophobic character of association. The ligands which bind at the active centers of the two enzymes (NAD^+, ATP, 3-phosphoglycerate) weakened the bienzyme association. Unlabeled 3-phosphoglycerate kinase was unable to displace the FITC-labeled enzyme from the complex. Taken together, the results indicate that interaction between D-glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase labeled by FITC is assisted by the dye, which may bind at nucleotide-binding sites of GPDH. No interaction was observed between the FITC-labeled 3-phosphoglycerate kinase and lactate dehydrogenase, which suggests that protein-protein interaction at specific "recognition" sites may be a prerequisite for the complex formation.

Fluorescein-5'-isothiocyanate (FITC), a fluorescent label capable of modifying lysine residues of a protein molecule, has been successfully used to detect specific protein-protein interactions between enzymes forming a complex. A number of complexes between glycolytic enzymes was characterized using this experimental approach [1-8]; recently Tressel et al. presented evidence for an interaction between L-threonine dehydrogenase and aminoacetone synthetase, employing FITC as a covalently bound conformation probe [9]. Meanwhile FITC is also known as a potent inhibitor of enzymes possessing nucleotide-binding sites.

Abbreviation: FITC, fluorescein-5'-isothiocyanate.

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Thus, it can specifically modify several ATPases, including sarcoplasmic reticulum Ca^{2+}-ATPase [10-12], microsomal Ca^{2+}-ATPase [13], Na^+, K^+-ATPases [14-16], as well as phosphorylase kinase [17] and pyruvate, phosphate dikinase [18]. In the latter case, FITC was shown to form a reversible complex with the enzyme prior to covalent modification of its ATP/AMP binding site. It seems therefore conceivable that FITC, like other fluorescent derivatives (such as tetraiodofluorescein and eosin [19-21]) is capable of non-covalently interacting with nucleotide-binding enzymes. In such a case, a possibility arises of an interaction between a dye molecule covalently bound to a protein, and another protein possessing an appropriate binding area. Thus, a complex can be formed wherein the two proteins interact "through" the dye molecule. As shown in the present paper, such a complex is probably formed between rabbit muscle D-glyceraldehyde-3-phosphate dehydrogenase and FITC-labeled rabbit muscle 3-phosphoglycerate kinase.

MATERIALS AND METHODS

FITC, NADH, D-glyceraldehyde-3-phosphate, Sepharose 4B, Tricine and dithiothreitol were purchased from Sigma (USA), EDTA, ATP, NAD^+ and porcine muscle lactate dehydrogenase were the products of Reanal (Hungary). All other chemicals were reagent grade commercial preparations. D-glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase were isolated from rabbit skeletal muscle and purified using affinity elution procedure of Scopes [22] with some modifications [23]. The dehydrogenase preparations contained bound NAD^+ (3-3.5 mole/tetramer), as determined by A_{280}/A_{260} ratio. To prepare the apoenzyme (A_{280}/A_{260} = 1.85), holo-glyceraldehyde-3-phosphate dehydrogenase solution in 10 mM sodium phosphate, 1 mM EDTA, pH 7.2 was treated with Norit A. Enzyme concentrations were determined spectrophotometrically using absorption coefficients $A_{280}^{0.1\%}$ of 1.0 and 0.83 for holo- and apo-enzymes respectively. An $A_{280}^{0.1\%}$ of 0.69 was used for 3-phosphoglycerate kinase [24]. Relative molecular masses of 140 000 and 47 000 were assumed for tetrameric D-glyceraldehyde-3-phosphate dehydrogenase and monomeric 3-phosphoglycerate kinase. D-glyceraldehyde-3-phosphate dehydrogenase activity measured in a mixture containing 100 mM sodium phosphate, 1.5 mM D-glyceraldehyde-3-phosphate, 2.0 mM NAD^+, 5 mM EDTA, pH 8.3 at 25°C, was 140-160 U/mg. Activity of 3-phosphoglycerate kinase was assayed at 25°C in a system containing 30 mM morpholinopropanesulphonic acid, 10 mM $MgSO_4$, 1 mM EDTA, 10 mM 3-phosphoglycerate, 3 mM ATP, 0.22 mM NADH and 8-10 U/ml D-glyceraldehyde-3-phosphate dehydrogenase, pH 7.2. It varied from 560 to 670 U/mg in different experiments. Fluorescence measurements were performed using a Hitachi MPF-4 spectrofluorimeter. 3-phosphoglycerate kinase labeling with FITC was carried out as follows. A crystalline suspension of the enzyme was centrifuged, and the pellet was dissolved in

10 mM Tricine, 1 mM EDTA, 0.5 mM FITC, pH 8.4 to obtain a final protein concentration of 0.05 mM. The mixture was kept in the dark at 4°C for 30 min; then the sample was filtered on a Sephadex G-50 column equilibrated with 10 mM sodium phosphate, 1 mM EDTA, pH 7.2, to remove unbound dye. The concentration of labeled protein was determined by the method of Bradford [25]. The extent of fluorescein labeling was determined by measuring the absorbance of the modified enzyme at 490 nm using an absorption coefficient of $6.6 \cdot 10^4$ $M^{-1}cm^{-1}$ [3]. The extent of labeling was found to vary in different experiments from 0.5 to 1.0 mol FITC/mol 3-phosphoglycerate kinase.

RESULTS

Fig. 1 shows that holo-D-glyceraldehyde-3-phosphate dehydrogenase is capable of quenching the fluorescence of FITC-labeled 3-phosphoglycerate kinase. Assuming that this process is due to an interaction between the enzymes, a second-order rate

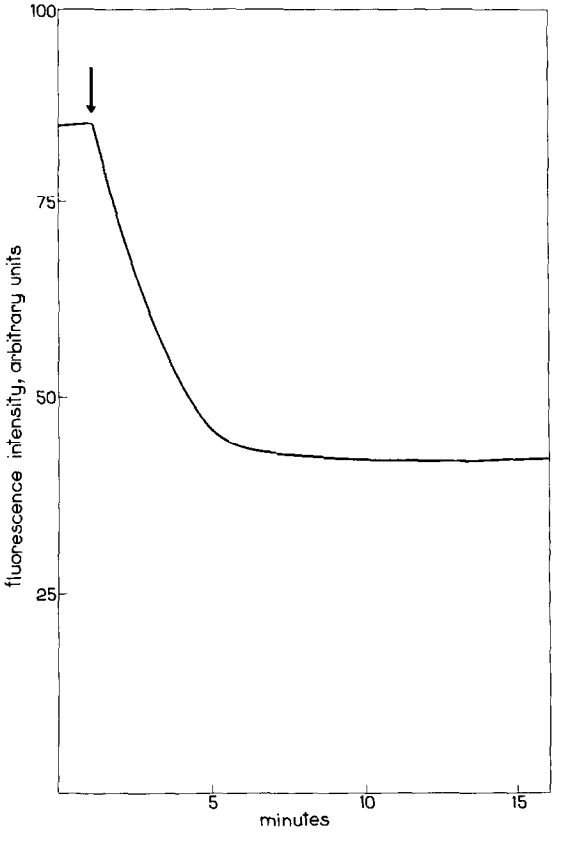

Fig. 1. Time-course of fluorescence quenching resulting from an interaction of FITC-labeled 3-phosphoglycerate kinase with D-glyceraldehyde-3-phosphate dehydrogenase. The labeled enzyme ($6.8 \cdot 10^{-7}$) was incubated in 10 mM sodium phosphate, 1 mM EDTA, pH 7.2 at 20°C. At time indicated by the arrow D-glyceraldehyde-3-phosphate dehydrogenase was added to give a final concentration of $6.0 \cdot 10^{-6}$M. Fluorescence was excited at 480 nm and measured at 520 nm.

constant of the bienzyme association was calculated from the dependence of a pseudo-first order rate constant of the fluorescence quenching on D-glyceraldehyde-3-phosphate dehydrogenase concentration. It was found to be $1.1\pm0.2\cdot10^{3}$ $M^{-1}s^{-1}$. To estimate the dissociation constant of the complex, a titration of FITC-labeled 3-phosphoglycerate kinase with D-glyceraldehyde--3-phosphate dehydrogenase was carried out. As seen in Fig. 2, apo- and holo-forms of the dehydrogenase exhibit different affinities for the FITC-labeled protein. The dissociation constants calculated from the data were found to be $3.2\cdot10^{-7}$ M for the apoenzyme and $6.25\cdot10^{-7}$ M for the holoenzyme. Shown on Fig. 3 are the results which demonstrate the effects of specific ligands (substrates of 3-phosphoglycerate kinase) on the fluorescence of FITC-labeled 3-phosphoglycerate kinase - D-glyceraldehyde-3-phosphate dehydrogenase mixture. One can see that 3-phosphoglycerate (1) and ATP (2) cause considerable increase in fluorescence intensity probably due to dissociation of the bienzyme complex. In the presence of saturating concentrations of both ligands added together (3), the fluorescence intensity reaches the initial level characteristic of uncomplexed FITC--labeled 3-phosphoglycerate kinase.

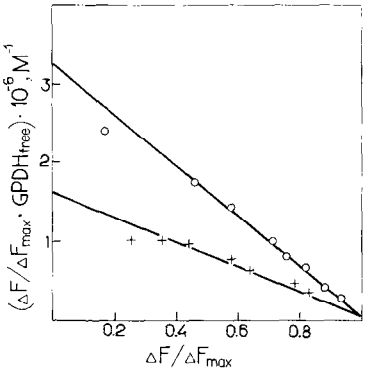

Fig. 2. The binding of a FITC-labeled 3-phosphoglycerate kinase with apo (o) and holo (+) forms of D-glyceraldehyde-3--phosphate dehydrogenase. A series of samples were prepared containing 10 mM sodium phosphate, 1 mM EDTA, $3.4\cdot10^{-7}$ M FITC--labeled 3-phosphoglycerate kinase and different amounts of D-glyceraldehyde-3-phosphate dehydrogenase (GPDH) in a final volume of 120 µl. The change of fluorescence intensity of a sample (ΔF) was measured after 30 min incubation at 20°C. Assuming that no more than one tetramer of GPDH binds with a FITC-labeled 3-phosphoglycerate kinase (PGK) molecule, a concentration of free GPDH can be calculated from the equation:

$$GPDH_{free} = GPDH_{total} - PGK_{total} \cdot \frac{\Delta F}{\Delta F_{max}}$$

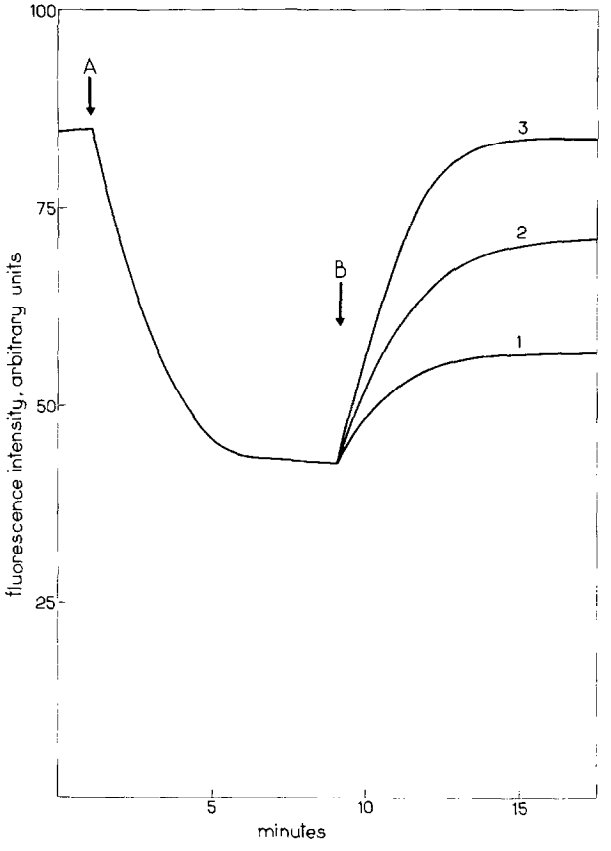

Fig. 3. Effect of specific ligands on fluorescence intensity of the mixture containing FITC-labeled 3-phosphoglycerate kinase and D-glyceraldehyde-3-phosphate dehydrogenase. $6.8 \cdot 10^{-7}$M FITC-labeled 3-phosphoglycerate kinase was incubated as indicated in Fig. 1; three samples were run parallel. At time indicated by the arrow A, $6 \cdot 10^{-6}$M D-glyceraldehyde-3-phosphate dehydrogenase was added to each of the samples. The arrow B indicates the addition of ligands to different samples. 1, 3-phosphoglyceric acid (final concentration, 10 mM); 2, ATP (final concentration, 3 mM); 3, ATP (final concentration, 3mM) plus 3-phosphoglycerate (final concentration, 10 mM). All additions were made in a volume of 3-5 µl; the final volume of a sample being 120 µl. The ligands added to the FITC-labeled 3-phosphoglycerate kinase alone caused only minor changes in fluorescence intensity (less than 7% of the maximal effect observed in the system containing D-glyceraldehyde-3-phosphate dehydrogenase).

We then examined the effect of temperature and ionic strength of the buffer on the association between the two enzymes. The results shown in Fig. 4, which demonstrate that the complex becomes stronger upon increase of the ionic strength of the medium, were unexpected in light of the large body of information existing in the literature on the factors which influence stability of multienzyme complexes. These data were also not consistent with our observations on the properties of

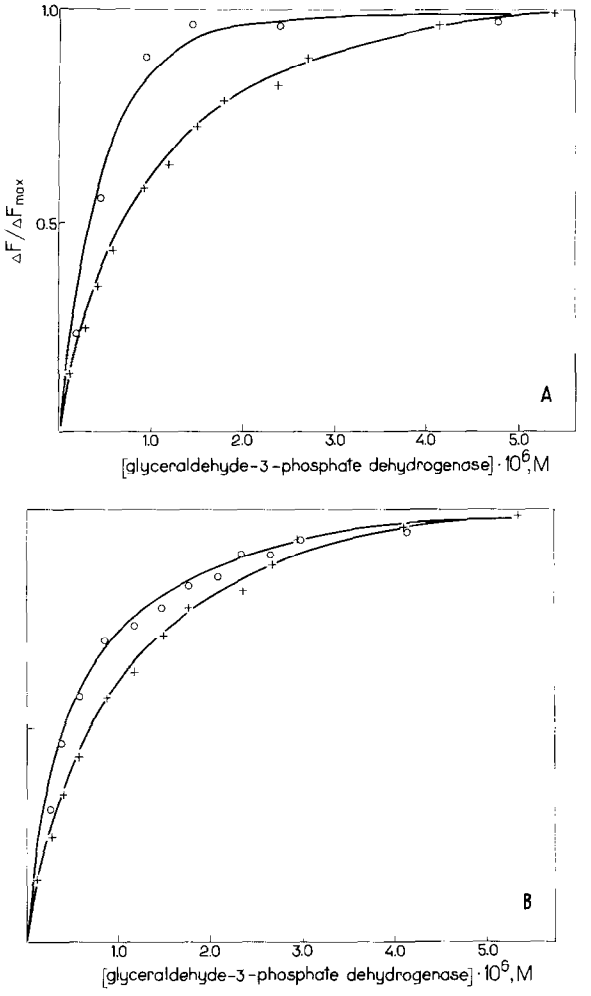

Fig. 4. Effect of ionic strength (A) and temperature (B) of the medium on the interaction between D-glyceraldehyde-3-phosphate dehydrogenase and FITC-labeled 3-phosphoglycerate kinase. Incubation was carried out as indicated in Fig. 1. A: FITC-labeled 3-phosphoglycerate kinase concentration, $3.4 \cdot 10^{-7}$M; (+) no additions, (o) with 170 mM NaCl. B: a series of samples containing FITC-labeled 3-phosphoglycerate kinase at a final concentration of $3.4 \cdot 10^{-7}$M and different amounts of D-glyceraldehyde-3-phosphate dehydrogenase were incubated at 20°C (+) or at 40°C (o) for 30 min and then fluorescence was measured in a thermostatically controlled unit of spectrofluorimeter.

complexes formed by unlabeled enzymes. We were, therefore, led to suggest that the fluorescent label somehow takes part in the bienzyme association. That supposition was supported by experimental evidence obtained in a study of the effect of unlabeled 3-phosphoglycerate kinase on the fluorescence ofthe bienzyme complex. In this series of experiments, the addition of an unlabeled kinase to a complex between D-glyceraldehyde-3-phospha-

te dehydrogenase and FITC-labeled 3-phosphoglycerate kinase did not influence the fluorescent characteristics of the complex. There was also no effect observed when unlabeled 3-phosphoglycerate kinase was present in 10-fold excess with respect to the FITC-labeled enzyme during the process of complex formation. The absence of competition between unlabeled and FITC-labeled-3-phosphoglycerate kinase for binding to the dehydrogenase was in line with the proposal that FITC attached to 3-phosphoglycerate kinase can interact with an appropriate area on the dehydrogenase molecule. The effects of specific ligands on the stability of the complex (see Figs 2 and 3) suggested that such interaction can occur at a nucleotide binding site of the active center. In this connection it seemed of interest to investigate the possibility of interaction between FITC-labeled 3-phosphoglycerate kinase and another enzyme possessing a nucleotide-binding region. This was done in experiments with lactate dehydrogenase used instead of D-glyceraldehyde-3-phosphate dehydrogenase. No complex formation could however be detected between this enzyme and 3-phosphoglycerate kinase labeled by FITC.

DISCUSSION

Several lines of evidence obtained in the present study indicate that FITC-labeled 3-phosphoglycerate kinase interacts with D-glyceraldehyde-3-phosphate dehydrogenase in a manner which is different from that typical for unlabeled enzymes. First, association between unmodified 3-phosphoglycerate kinase and D-glyceraldehyde-3-phosphate dehydrogenase observed in our previous studies was normally instantaneous [26], i.e. a much faster process than that observed with a FITC-labeled enzyme. Second, specific ligands (coenzymes and substrates) increased the mutual affinity of the enzymes [23,31], in contrast to the results obtained in this investigation (Figs 2,3). Third, the effect of ionic strength on the stability of a bienzyme complex observed in the present work is opposite to the one obtained with unlabeled proteins (our unpublished observations and data from literature [32,33]). Fourth, unlabeled 3-phosphoglycerate kinase was unable to displace FITC-labeled enzyme from a complex.

Yet, we do not believe that the mode of interaction between FITC-labeled 3-phosphoglycerate kinase and D-glyceraldehyde-3-

-phosphate dehydrogenase is non-specific. In fact, no interaction was observed when muscle lactate dehydrogenase was used instead of D-glyceraldehyde-3-phosphate dehydrogenase. This leads us to assume that some specific interaction between the two enzymes is needed before a relatively stable complex can be formed. Taking into consideration that this process is rather slow, we speculate that it may include two steps: a fast protein-protein association occuring at specific sites involved in complex formation between unmodified proteins, and a slow process of a rearrangement whereupon the dye is introduced into a nucleotide-binding region of D-glyceraldehyde-3--phosphate dehydrogenase. Previously we have shown a number of fluorescent probes (1-anilino-8-naphthalene sulphonate, auramine O, acridine orange) to be capable of binding with D-glyceraldehyde-3-phosphate dehydrogenase at specific sites located at the active site region or in its proximity [27,28]. The results of the present study are consistent with the idea that fluorescein moiety can also be bound at these sites. Since the dye remains covalently linked to 3-phosphoglycerate kinase, the above interaction would stabilize the bienzyme complex.

Muscle lactate dehydrogenase which is also capable of binding fluorescent probes at specific sites [29,30] cannot, however, form a complex with FITC-labeled 3-phosphoglycerate kinase. This suggests that specific protein-protein interactions are a prerequisite for bienzyme complex formation and that no such interactions can arise between lactate dehydrogenase and 3-phosphoglycerate kinase.

Some concluding remarks are needed in the way of comparing our data with the results obtained in other laboratories with the use of FITC as a fluorescent label to study protein-protein interactions. In a series of papers by Ovadi et al. [3-6], as well as in studies by Batke et al. [8] and Gavilanes et al.[2], interactions between a FITC-labeled enzyme with another protein were followed by measuring the anisotropy or polarization of fluorescence of the dye. The authors specify that under the experimental conditions employed no change in the fluorescence quantum yield of the label was observed in the course of interaction between a FITC-labeled enzyme and an unlabeled protein [2-5], which indicates that no interaction through the dye molecule occurred. The absence of non-specific effects in these

studies can probably be explained by the fact that the oligomeric enzymes employed to form the complexes were only partially labeled (often no more than 50% of the subunits within a macromolecule contained FITC [4-7]). Under such conditions, unlabeled subunits of different enzymes could interact forming a complex. However, other explanations of the observed phenomena are not excluded.

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