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HETEROLOGOUS ENZYME-ENZYME COMPLEX BETWEEN D-FRUCTOSE-1,6-BISPHOSPHATE ALDOLASE AND TRIOSEPHOSPHATE ISOMERASE FROM *CERATITIS CAPITATA* *

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Evidence is reported for the interaction between D-fructose-1,6-bisphosphate aldolase (D-fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13) and triosephosphate isomerase (D-glyceraldehyde-3-phosphate ketol-isomerose, EC 5.3.1.1) from flight muscle of the insect, *Ceratitis capitata*.

There is considerable discussion about the possibility that the enzymes of glycolysis form a structurally integrated system in vivo [1–3]. Physicochemical evidence for interaction between D-fructose-1,6-bisphosphate aldolase and D-glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle and for interaction between fructose-1,6-bisphosphatase and D-fructose-1,6-bisphosphate aldolase from rabbit liver has been reported [4,5]. A multienzyme complex containing all the glycolytic enzyme activities has been demonstrated in extracts of *Escherichia coli* [6].

Flight muscle fibers of insects are remarkable in their intense respiratory activity and represent a very interesting tissue in the study of bioenergetic metabolism and biological oxidation control [7]. Individual enzymes of the glycolytic pathway of the flight muscle of the insect, *Ceratitis capitata*, have been previously studied [8–10].

In the present paper, physicochemical evidence for the interaction between D-fructose-1,6-bisphosphate aldolase and triosephosphate isomerase from this insect is presented.

D-Fructose-1,6-bisphosphate aldolase (D-fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13) and triosephosphate isomerase (D-glyceraldehyde-3-phosphate ketol isomerase EC 5.3.1.1) were purified from *Ceratitis capitata* flies (Wiedeman) to apparent electrophoretic homogeneity as previously described [9,10]. D-Glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate : NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12) from yeast was purchased from Sigma Chemical Co. Ultragel AcA 44 was obtained from LKB. All other reagents were high purity commercial samples from Merck A.G., Sigma, Boehringer and Pharmacia.

Enzyme concentrations were determined spectrophotometrically at 280 nm [9,11,12]. The following values were used to calculate enzyme concentration: for aldolase, M_r = 160 000 and specific activity = 1 760 mol fructose 1,6-bisphosphate/mol enzyme per min [9]; for isomerase, M_r = 55 000 and spec. act. = 121 000 mol glyceraldehyde 3-phosphate/mol enzyme per min [11]; for dehydrogenase, M_r = 145 000 and spec. act. = 12 000 mol NADH/mol enzyme per min [4,12]. Enzyme activities were determined at 25°C as previously described [4,0,10].

The isomerase ($2 \cdot 10^{-6}$ M) was labeled with fluorescein isothiocyanate (0.1 mM) in 50 mM Tris-HCl buffer, pH 8.5, containing 1 mM EDTA and 3

* This work is dedicated to Professor Alessandro Rossi Fanelli on the occasion of his seventy-fifth birthday.

mM glyceraldehyde 3-phosphate. The mixture was incubated for 1.5 h in the dark at 4°C. Free fluorescent dye was then removed from the protein by gel filtration through a Sephadex G-25 (Pharmacia) column equilibrated with 50 mM Tris-HCl (pH 7.5)/1 mM EDTA. The latter procedure removed all traces of free dye from the protein-conjugated dye, as demonstrated by a second chromatography through the same column in which the protein peak was coincident with the fluorescent peak. The degree of labeling, determined spectrophotometrically [4], was 1.5 ± 0.5 mol dye per mol isomerase. The labeled enzyme retained, within the experimental error, all the original activity.

The intensity and polarization fluorescence and the anisotropy of labeled isomerase were determined as previously described [4] in 50 mM Tris-HCl/1 mM EDTA, pH 7.5, at 25°C.

Gel filtration equilibrium was performed according to published procedures [13]. 0.3 ml packed Ultrogel AcA 44, washed three times with 50 mM Tris-HCl (pH 7.5)/1 mM EDTA, was mixed with 0.2 ml of the same buffer containing aldolase, isomerase, dehydrogenase, mixtures of these enzymes, or blue dextran (Pharmacia). The suspensions were stirred for 2 h at 25°C. At the end of this time, the suspensions were centrifuged and aliquots of the clear supernatant were removed for determination of enzyme activity or blue dextran concentration. Control experiments demonstrated that gel filtration equilibrium was reached during the incubation of the suspension at 25°C. The distribution coefficient was defined [13] as

$$K_D = (V_f - V_a)/(V_t - V_a)$$

where V_f is the penetration volume of the enzyme (total units of enzyme added divided by units of enzyme per ml in aqueous phase), V_a is the volume of the aqueous phase (total blue dextran added divided by concentration of blue dextran in the aqueous phase), and V_t is the total volume of the system. V_a was equal to 0.12 ± 0.01 ml.

In the presence of D-fructose-1,6-bisphosphate aldolase (ranging from $3.7 \cdot 10^{-7}$ to $8 \cdot 10^{-6}$ M), the anisotropy of fluorescein-labeled triosephosphate isomerase ($8 \cdot 10^{-8}$ M) increased rapidly and reached a constant value after 1 min of incubation of the enzymes at 25°C. The anisotropy approached a limiting value of 0.17 with excess aldolase (Fig. 1).

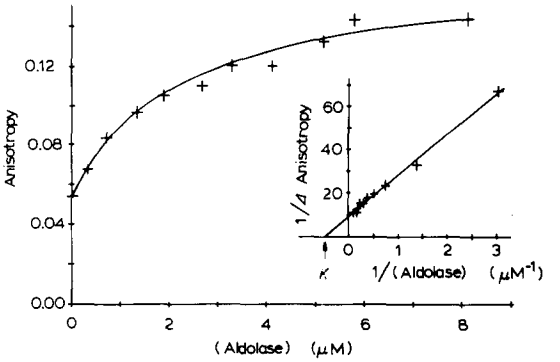

Fig. 1. Anisotropy of fluorescein-isothiocyanate-isomerase ($8 \cdot 10^{-8}$ M), measured after it had reached a constant value, as a function of aldolase concentration in 50 mM Tris-HCl/1 mM EDTA, pH 7.5, at 20°C. Inset: double-reciprocal plot of the change in anisotropy against aldolase concentration.

Free fluorescent dye was not detectable in the preparation of labeled isomerase. Moreover the quantum yield of fluorescein-isomerase conjugates

TABLE I

DISTRIBUTION COEFFICIENT (K_D) OF ALDOLASE AND TRIOSEPHOSPHATE ISOMERASE FROM *CERATITIS CAPITATA* IN ULTROGEL AcA 44

The amount of isomerase present in the assays is 0.13 nmol.

Enzyme (nmol)		K_D	
Aldolase	Glyceraldehyde-3-phosphate dehydrogenase	Triosephosphate isomerase	Aldolase
–	–	0.56	–
–	0.104	0.56	–
–	0.210	0.56	–
0.050	–	0.43	0.46
0.092	–	0.38	0.45
0.123	–	0.42	0.43
0.239	–	0.37	0.41
0.323	–	0.29	0.40
0.500	–	0.26	0.40
0.625	–	0.27	0.40
0.773	–	0.25	0.39
0.938	–	0.20	0.37
0.123	–	–	0.47 [a]

[a] Determined in absence of isomerase.

was not appreciably affected by the presence of aldolase. These findings appear to exclude the possibility that the transfer of any non-covalently bound fluorescein from isomerase to aldolase could play any part and that aldolase binding was due to the fluorescein moiety.

The above considerations suggest that the observed increase in anisotropy is related to the binding of aldolase to isomerase. This hypothesis was confirmed by gel filtration equilibrium experiments.

As shown in Table I, the distribution coefficient of isomerase (0.13 nmol in 0.5 ml of gel suspension) in Ultrogel AcA 44 decreased upon addition of aldolase (from 0.05 to 0.938 nmol in 0.5 ml of gel suspension) to the system. Isomerase concentration was determined measuring its activity in the aqueous phase of the gel suspension. Control experiments, performed without Ultrogel, revealed that isomerase is completely stable under these conditions and, when the enzymes are present in the low levels used in assay, aldolase does not alter isomerase activity. Moreover, the Ultrogel AcA 44 distribution coefficient of aldolase measured in the presence of isomerase was lower than that measured in the absence of isomerase. Finally, D-glyceraldehyde-3-phosphate dehydrogenase (up to 0.21 nmol of enzyme in 0.5 ml of gel suspension), a protein with molecular weight similar to that of aldolase [9,12], did not decrease the distribution coefficient of isomerase in Ultrogel.

Therefore, using aldolase concentrations similar to those employed in the fluorescence polarization experiments reported above, it is possible to demonstrate that aldolase decrease the ability of isomerase to penetrate Ultrogel AcA 44. The formation of a macromolecular complex between D-fructose-1,6-bisphosphate aldolase and triosephosphate isomerase purified from flight insect *Ceratitis capitata* is strongly suggested. The apparent association constant ($K = 4.7 \cdot 10^5\ M^{-1}$) for this complex can be calculated [4,14] from the double-reciprocal plot reported in Fig. 1 inset.

Present results suggest that D-fructose-1,6-bisphosphate aldolase and triosephosphate isomerase may reversibly interact in vitro. Other glycolytic and gluconeogenic enzymes have also been shown to interact in vitro [4,5]. Tempting though they be, extrapolations about the possible physiological significance of these findings should be made with great caution because of the different and not completely known physiological conditions existing within the cell.

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