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Kinetic pathways of formation and dissociation of the glycerol-3-phosphate dehydrogenase–fructose-1,6-bisphosphate aldolase complex

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Quantitative analysis of the time courses of fluorescence anisotropy changes due to the binding of fructose-1,6-bisphosphate aldolase to the dissociable cytoplasmic glycerol-3-phosphate dehydrogenase covalently labelled with fluorescent dye was carried out. The behaviour of the aldolase–dehydrogenase system seems to be consistent with a cyclic reversible model characterized by the formation and dissociation of complexes of both the monomeric and the dimeric forms of dehydrogenase with aldolase, and rapid equilibrium between the free monomeric and dimeric forms of dehydrogenase. The half-life time of the formation of dimeric dehydrogenase–aldolase complex at the concentration of the enzymes expected to exist in the cell (i.e. in the micromolar range) is some minutes, and the time needed for equilibration between the aldolase-bound dimeric and monomeric forms of dehydrogenase is a few minutes as well. Consequently, one may expect that both the formation and the dissociation of this heterologous enzyme complex have physiological relevance.

Complex-formation and functional interactions between fructose-1,6-bisphosphate aldolase (EC 4.1.2.13) and glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) have been intensively studied. Myogen, a sarcoplasmic preparation rich in glycolytic enzymes, forms a single slow-moving peak in boundary electrophoresis (Amberson *et al.*, 1964); one of its fractions (myogen A), which contains aldolase and glycerolphosphate dehydrogenase, crystallizes readily and seems to be homogeneous in sedimentation-equilibrium analysis (Baranowski & Niederland, 1949). More-recent evidence from active-enzyme-band centrifugation and stopped-flow kinetic analysis suggested that fructose-1,6-bisphosphate aldolase formed a complex with glycerolphosphate dehydrogenase and that in this complex dehydrogenase activity was enhanced (Batke *et al.*, 1980).

In a previous paper we reported the quantitative analysis of interaction at equilibrium between aldolase and glycerolphosphate dehydrogenase labelled with fluorescent dye by using a fluorescence-polarization technique. It was found that the fluorescence anisotropy of the fluorescein-dye-labelled dehydrogenase increased with time on the addition of unlabelled aldolase approaching a limit value (Ovádi *et al.*, 1983). This fact has been attributed to the binding of aldolase to dehydrogenase. Study of the concentration-dependence of the specific activity of dehydrogenase (Batke *et al.*, 1980) suggested that the functioning dimeric dehydrogenase dissociated into the less-active monomers. The dissociation constant for the dimer–monomer equilibrium was also estimated from the enzyme-concentration-dependent anisotropy curves (Ovádi *et al.*, 1983). To characterize quantitatively the equilibria between the two enzymes, a model was developed based on the assumption that both the dimeric and the monomeric forms of dehydrogenase interact with aldolase with a 1:1 stoichiometry. In the present work we studied the kinetics of the formation of complexes between monomeric and dimeric forms of glycerolphosphate dehydrogenase and aldolase, and developed a cyclic reversible model that seems to be consistent with the behaviour of this enzyme system.

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Materials and methods

Experiments were carried out with four-times-recrystallized glycerolphosphate dehydrogenase

and aldolase prepared from rabbit skeletal muscle (Telegdi, 1964; Taylor *et al.*, 1948). Fluorescein isothiocyanate–Celite was from Fluka (Buchs, Switzerland); all other chemicals were commercial preparations of reagent grade from Reanal (Budapest, Hungary). The concentrations of the enzymes were measured spectrophotometrically at 280nm by using the coefficients $A_{280}^{0.1\%}=0.73$ and $A_{280}^{0.1\%}=1.0$ for aldolase and glycerolphosphate dehydrogenase respectively. The concentration of dehydrogenase is given in dimeric equivalents. The specific activities of the enzymes, determined as described previously (Batke *et al.*, 1980), were 40–45kat/mol for aldolase and 35–40kat/mol for monomeric glycerolphosphate dehydrogenase. All experiments were carried out in 50mM-Tris/HCl buffer containing 1mM-EDTA, final pH7.5, at 20°C.

Fluorescein isothiocyanate labelling of glycerolphosphate dehydrogenase was carried out as described previously (Ovádi *et al.*, 1983). The degree of the labelling was between 0.5 and 1.5mol per mol of dimer dehydrogenase, and the value of anisotropy was independent of the degree of labelling. The covalent binding of fluorescein dye up to 2mol/mol of dimeric enzyme did not influence the catalytic activity (Ovádi *et al.*, 1983).

Fluorescence polarization was measured in an Applied Photophysics SP 3 instrument at an excitation wavelength of 470nm. The polarization was calculated by using the relation:

$$p=(V-GH)/(V+GH)$$

where V and H denote vertically and horizontally polarized fluorescence intensities at 520nm and G is the instrumental correction (Rank, 1947). The anisotropy was calculated from the relation:

$$A=2p/(3-p)$$

The error of determination of anisotropies is 5%.

Evaluation of experiments, theoretical calculations and curve-fitting were performed with the aid of Hewlett–Packard 9825A programmable calculator and a 9872A plotter.

Best parameters in curve fittings were achieved by a non-linear least-squares procedure, based on a modified sequential simplex algorithm of Spendley *et al.* (1962).

The objective function is:

$$\psi^2=\sum_{i=1}^{n}\psi_i^2=\sum_{i=1}^{n}\left(\frac{y_i-y_{ri}}{y_i}\right)^2$$

where $y_{ri}=y_r(x_i, p_j)$ is the regression function with n_p parameters, p_j $(j=1, \ldots n_p)$, for a set of n data pairs x_i, y_i. The x and y correspond to the time and anisotropy respectively. The goodness of fit was tested by Fisher test (Sokal & Rohlf, 1969). The explained variance:

$$S_e=(n_p-1)^{-1}\cdot\sum_{i=1}^{n}(y_{ri}-\bar{y})^2$$

(where $\bar{y}=n^{-1}\cdot\sum_{i=1}^{n}y_i$) must be larger than the unexplained mean square:

$$S_u=(n-n_p)^{-1}\cdot\sum_{i=1}^{n}(y_i-y_{ri})^2$$

so the fit will be accepted with $1-\alpha$ significance level if the ratio S_e/S_u exceeds the critical value $F_{\alpha(n_p-1,\ n-n_p)}$. Two regression curves were considered different if significant difference exists between the mean values of the residuals in the conventional Student's t test.

Results and discussion

In our previous work (Ovádi *et al.*, 1983) evidence was presented for the simultaneous binding of tetrameric aldolase to monomeric and dimeric glycerolphosphate dehydrogenase, and the dissociation constants of the free dimeric dehydrogenase (K), aldolase-bound monomeric dehydrogenase (K_1) and aldolase-bound dimeric dehydrogenase (K_2) were estimated. In order to predict the kinetic pathways and rates of formation and dissociation of dehydrogenase and aldolase complexes at concentrations expected to exist under physiological conditions, we have carried out a detailed kinetic analysis of this system. In various sets of experiment the time courses of increase of anisotropy of dehydrogenase labelled covalently with fluorescein isothiocyanate in the presence of unlabelled aldolase were recorded at different enzyme concentrations. Aldolase was always used in excess to ensure pseudo-first-order conditions. We have found that the reaction follows first-order kinetics when the concentration of labelled dehydrogenase is relatively low (0.2μM) with respect to the dissociation constant (K). Under this condition the monomeric form of dehydrogenase is the predominant form and the increase in anisotropy can be attributed effectively only to the complex-formation between monomeric dehydrogenase and aldolase, as expected from the equilibrium measurements (Ovádi *et al.*, 1983). However, at dehydrogenase concentrations where the equilibrium concentrations of dimeric and monomeric dehydrogenase with different specific anisotropies are comparable the reaction does not follow single first-order kinetics even if aldolase is in excess. At least two processes may be superimposed, namely the binding of monomeric and dimeric glycerolphosphate dehydrogenase to aldolase resulting in

complexes with different specific anisotropies (Table 1). (Ovádi *et al.*, 1983). To characterize kinetically this two-enzyme system, the following possible model was developed based on the assumption that the monomer and dimer forms of free dehydrogenase are in rapid equilibrium with respect to heterologous complex-formation. This assumption is supported by the time course of decrease of anisotropy of labelled dimeric dehydrogenase on dilution due to the dissociation of dimeric enzyme into monomers (see below):

$$M + M \underset{k_-}{\overset{k_+}{\rightleftharpoons}} D \qquad K = \frac{[M]^2}{[D]}$$

$$M + A \underset{k_1K_1}{\overset{k_1}{\rightleftharpoons}} MA \qquad K_1 = \frac{[M][A]}{[MA]}$$

$$D + A \underset{k_2K_2}{\overset{k_2}{\rightleftharpoons}} DA \qquad K_2 = \frac{[D][A]}{[DA]}$$

$$MA + M \underset{k_3}{\overset{k_3/K_3}{\rightleftharpoons}} DA \qquad K_3 = \frac{[MA][M]}{[DA]}$$

where D and M are the dimeric and monomeric forms of free glycerolphosphate dehydrogenase respectively, and MA and DA are the complexes of the corresponding dehydrogenase forms with aldolase. The values of the dissociation constants K_1, K_2, K_3 and K were taken from the equilibrium measurements (Ovádi *et al.*, 1983); k_1 and k_2 are second-order rate constants, and k_3 is a first-order rate constant.

For the proposed model, the following relationships hold:

$$\frac{d[D]}{dt} = -k_2[A][D] + k_2K_2[DA] - k_-[D] + k_+[M]^2$$

$$\frac{d[M]}{dt} = -k_1[A][M] + k_1K_1[MA] + 2k_-[D] - 2k_+[M]^2 + k_3[DA] - \frac{k_3}{K_3}[M][MA]$$

$$\frac{d[DA]}{dt} = -k_2K_2[DA] + k_2[A][D] - k_3[DA] + \frac{k_3}{K_3}[MA][M]$$

$$\frac{d[MA]}{dt} = -k_1K_1[MA] + k_1[A][M] - \frac{k_3}{K_3}[MA][M] + k_3[DA]$$

where $k_-[D] = k_+[M]^2$, since rapid equilibrium exists. The relations of the enzyme concentrations are:

$$[\text{Aldolase}]_{\text{total}} \sim [A] \gg [D] = \frac{[M]^2}{K}$$

$$[\text{Glycerolphosphate dehydrogenase}]_{\text{total}} = [D] + \frac{[M]}{2} + [DA] + \frac{[MA]}{2}$$

The observed anisotropy is the weighted sum of the specific anisotropies of M, D, MA and DA:

$$\text{Anisotropy (observed)} = \frac{2a_D[D] + a_M[M] + 2a_{DA}[DA] + a_{MA}[MA]}{2[D] + [M] + 2[DA] + [MA]}$$

where a_D, a_M, a_{MA} and a_{DA} are the specific anisotropies of the corresponding species taken from the equilibrium measurements (Ovádi *et al.*, 1983). The fluorescence quantum efficiencies of the different protein complexes are not different (Ovádi *et al.*, 1983). The time course of changes in anisotropy due to the addition of unlabelled aldolase was measured at different dehydrogenase concentrations in the range 0.2–2.4 μM. Unlabelled aldolase at three different concentrations was added to each dehydrogenase sample. Aldolase was in excess (10–30-fold) with respect to labelled dehydrogenase.

Two of the four differential equations were solved by Euler's method, since the others could be eliminated by taking into account the rapid equilibrium between the forms D and M and the law of mass conservation. The time courses are related with the constraint that $[MA] = [DA] = 0$ at $t = 0$.

Evaluation of the proposed model was carried out by an iterative program of weighted least-square evaluation of k_1, k_2 and k_3, as described in

the Materials and methods section, by assuming rapid equilibrium between M and D. The mean values ± S.E.M. for k_1, k_2 and k_3, from 16 sets of experiments were given to be $(2.0\pm0.5)\times 10^3\,M^{-1}\cdot min^{-1}$, $(2.0\pm0.5)\times 10^4\,M^{-1}\cdot min^{-1}$ and $0.4\pm0.1\,min^{-1}$ respectively. The computations of two typical time courses of anisotropy change are presented in Fig. 1. With the optimal parameter set, the goodness of fit of the individual time courses was significant at the 1% level in all series of experiment. A t test was applied to check whether the replacement of the best-fitting parameter sets with the average values of these parameters influenced the average residual error significantly.

We have found the difference to be not significant at the 20% level. This means that the average of parameter sets is appropriate for the fitting of all the time courses of anisotropy changes at various enzyme concentrations.

We have analysed whether the assumption that the monomer and dimer forms of free dehydrogenase are in rapid equilibrium with respect to heterologous complex-formation is valid or not.

We observed that, if 12 μM labelled dehydrogenase was diluted 60-fold, the decrease of anisotropy due to dilution was too rapid for us to follow the complete time course by the technique applied. However, we could estimate that at least 95% of the total change of anisotropy took place in less than 0.5 min. From these experiments a lower limit to the association rate constant, k_+, can be deduced as follows. Introducing the notations:

$$M_0 = \frac{4E}{1+\sqrt{1+h\dfrac{16E}{K}}}$$

and

$$\bar{M} = \frac{4E}{1+\sqrt{1+\dfrac{16E}{K}}}$$

where M_0 and $\bar{M}$ are the initial and final concentrations of monomer (in dimer equivalents) respectively, E is the total concentration of labelled dehydrogenase after dilution and h is the dilution factor.

$$K = \frac{k_-}{k_+} = 4\,\mu M$$

Then

$$k_+ = \frac{1}{qt}\cdot \ln\left(\frac{\dfrac{s}{100}\cdot\dfrac{2}{q}+1}{\dfrac{\bar{M}-M_0}{\bar{M}}-2}\Big/\left(1-\frac{s}{100}\right)\right)$$

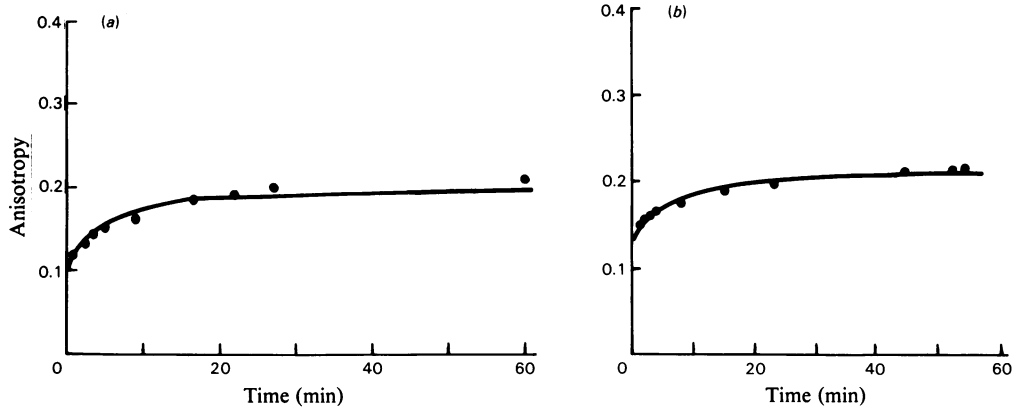

Fig. 1. *Time courses of anisotropy change of fluorescein-dye-labelled glycerolphosphate dehydrogenase in the presence of excess of unlabelled aldolase in 50 mM-Tris/HCl containing 1 mM-EDTA, final pH 7.5*

(*a*) The concentrations of labelled dehydrogenase and aldolase were 0.21 μM and 26 μM respectively. (*b*) The concentrations of labelled dehydrogenase and aldolase were 0.96 μM and 13.0 μM respectively. The continuous lines were computed by the optimal parameter sets: for (*a*), $k_1 = 2\times10^3\,M^{-1}\cdot min^{-1}$, $k_2 = 2.5\times10^4\,M^{-1}\cdot min^{-1}$, $k_3 = 0.5\,min^{-1}$; for (*b*), $k_1 = 2\times10^3\,M^{-1}\cdot min^{-1}$, $k_2 = 1.5\times10^4\,M^{-1}\cdot min^{-1}$; $k_3 = 0.3\,min^{-1}$; in accordance with the reversible cyclic model assuming rapid equilibrium between M and D.

Table 1. *Kinetic parameters of the glycerolphosphate dehydrogenase (GDH) and aldolase interacting system*

The values in columns 2, 3 and 4 were taken from our previous papers (Batke *et al.*, 1980; Ovádi *et al.*, 1983). The half-life times of the processes of complex-formation were calculated from the pseudo-first-order rate constants at 10 μM-aldolase concentrations.

Enzyme system	Specific activity (kat/mol)	Specific anisotropy	Dissociation constant (μM)	For association: Second-order rate constant ($M^{-1} \cdot min^{-1}$)	For association: Half-life time at 10 μM-aldolase (min)	For dissociation: First-order rate constant (min^{-1})	For dissociation: Half-life time (min^{-1})
GDH monomer	30	0.05 ± 0.02	4	$> 1.1 \times 10^6$		> 4.6	
GDH dimer	400	0.25 ± 0.05					
Complex with aldolase							
GDH monomer	?	0.2 ± 0.02	1	$(2.0 \pm 0.5) \times 10^3$	34.5	2.0×10^{-3}	345
GDH dimer	800	0.32 ± 0.02	0.2	$(2.0 \pm 0.5) \times 10^4$	3.5	4.0×10^{-3}	173
MA + M ⇌ DA			0.8	5.0×10^5		0.4 ± 0.1	1.7

where $q = K \cdot \sqrt{1 + 16E/K}$ and t is the time at which the anisotropy has decreased to a fraction $s/100$ of the total change.

With the experimentally obtained data presented above ($s \geqslant 95$; $t \leqslant 0.5$ min) we get the lower limits for rate constants of dimerization (k_+) and of dissociation ($k_- = k_+ K$). These are $k_+ > 1.15 \times 10^6 M^{-1} \cdot min^{-1}$ and $k_- > 4.60 min^{-1}$ (Table 1).

At 0.96 μM and 13 μM concentrations of dehydrogenase and aldolase respectively, presented in Fig. 1(*b*) as an example, we have found that:

$$k_+[M] = 0.82 min^{-1} \gg k_1[A] = 0.03 min^{-1}$$

and

$$k_- = 4.60 min^{-1} \gg k_2[A] = 0.26 min^{-1}$$

Therefore both dimerization and dissociation are in rapid equilibrium with respect to the rate of formation of heterologous complexes. This is valid for all the sets of experiments.

Furthermore, it should be emphasized that the formal fitting of the anisotropy changes, due to the complex-formation, by a single exponential curve has no physical meaning when both the free forms (M and D) and complexed forms of dehydrogenase (MA and DA) have different specific anisotropy values (Table 1) even if aldolase is in excess in all cases.

In addition, two points should be made, as follows. (i) If it was assumed that the heterologous enzyme complexes (MA and DA) formed irreversibly, similar values of parameters were found to simulate the time courses. This observation is not surprising, since in both sets of experiments aldolase was in excess, and thus practically no free dehydrogenase might have existed at the final equilibrium state. Nevertheless, since we have already demonstrated the reversibility of complex-formation (Ovádi *et al.*, 1983), this finding must be incorporated in the kinetic model. (ii) If no direct conversion between heterologous enzyme complexes MA and DA was assumed (non-cyclic model), the time courses measured at different glycerolphosphate dehydrogenase concentrations could be fitted only by different sets of parameters. Therefore we can conclude that our system is consistent with the proposed reversible cyclic model as the simplest one that is consistent with our previous finding, namely that both forms of the dissociable dehydrogenase can form complexes with aldolase (Ovádi *et al.*, 1983).

Table 1 summarizes the quantitative data referring to both homologous and heterologous interactions of glycerolphosphate dehydrogenase and aldolase. It is clear that the specific activity of the dimeric dehydrogenase is significantly higher than that of its dissociated form. The earlier

observation that the activity of the dehydrogenase is enhanced as a result of the complex-formation with aldolase has been attributed to a shift in the dimer–monomer equilibrium of dehydrogenase as well as to an aldolase-induced conformational change in the dimeric dehydrogenase (Batke *et al.*, 1980). Indeed, physicochemical investigations (Ovádi *et al.*, 1983) have suggested that both monomeric and dimeric forms of glycerolphosphate dehydrogenase can interact with aldolase, and the affinity of the dimeric dehydrogenase to aldolase is 5-fold higher than that of the monomeric one. The present data indicate that the rate of complex-formation between monomeric glycerolphosphate dehydrogenase and aldolase is rather slow. Similarly, slow complex-formation between other 'soluble' enzymes has been already observed with aspartate aminotransferase and glutamate dehydrogenase (Salerno *et al.*, 1975), aldolase and glyceraldehyde-3-phosphate dehydrogenase (Ovádi *et al.*, 1978; Grazi & Trombetta, 1980) and aldolase and triosephosphate isomerase (Salerno & Ovádi, 1982). However, in the present case the second-order rate constant of the formation of the dimeric glycerolphosphate dehydrogenase–aldolase complex is at least one order of magnitude higher than that of monomeric dehydrogenase–aldolase complex. Thus it can be surmised that the heterologous complex at aldolase (45 μmol/kg of tissue) and glycerolphosphate dehydrogenase (4 μmol/kg of tissue) concentrations expected to exist in the cell (Srere, 1967) is formed preferentially between dimeric glycerolphosphate dehydrogenase and aldolase with a half-life time of a few minutes, ensuring the higher catalytic efficiency for the functionally coupled enzyme system. Moreover, the formation of the monomeric dehydrogenase–aldolase complex from DA complex is even quicker than that from M and A. The complex-formation, which corresponds to the final equilibrium state, may occur with a half-life time of a few minutes (cf. Table 1).

Taking the two experimentally determined values (dissociation constants and second-order rate constants of the complex-formation), one can arrive by simple calculation at half-life times for the dissociation of complexes into individual enzymes of several hours (Table 1). The dissociation process of both complexes of monomeric and dimeric glycerolphosphate dehydrogenase with aldolase is too slow for the attribution of any physiological importance to the dissociation.

However, if we consider the DA→MA+M process the half-life time is significantly lower than that of the dissociation of heterologous complexes into individual enzymes. Hence we can hypothesize that, if either or both enzyme concentrations decrease [for example, owing to the complex-formation with other enzyme(s)], the dissociation of the complex goes through the following steps:

DA→MA+M	$t_{\frac{1}{2}}$: 1.7 min
M+M⇌D	Rapid equilibrium
D+A→DA	$t_{\frac{1}{2}}$: 3.5 min at 10 μM-aldolase

whereas the dissociation of monomeric dehydrogenase–aldolase complex is much slower:

MA→M+A	$t_{\frac{1}{2}}$: 173 min

Therefore the system can reach the new equilibrium state within a few minutes. This time range is still long with respect to the catalytic turnover number of these enzymes; however, it is comparable with the time range of a regulation process, e.g. in the case of hysteretic enzymes (Frieden, 1979).

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