DETERMINATION OF THE KINETIC PARAMETERS OF PHOSPHOFRUCTOKINASE DISSOCIATION

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1. Introduction

It has been demonstrated that phosphofructokinase from rabbit skeletal muscle is dissociated into inactive subunits in the presence of low enzyme concentrations [1]. Dissociation constants of about 10^{-6} M, as estimated from computer simulations [2–5], suggested physiological significance of the dissociation process in the regulation of cellular phosphofructokinase activity. In this study, the determination of rate and equilibrium constants of the dissociation of phosphofructokinase is described. The kinetic method used in the experiments takes advantage of the enhancement of the fluorescence of 2-[*N*-methylanilino] naphthalene 6-sulphonate (MNS) upon dissociation of the enzyme [4].

2. Materials and methods

Phosphofructokinase was purified and assayed as described previously [6]. MNS was synthesized according to Cory et al. [7]. The determination of kinetic parameters was performed with a Perkin–Elmer MPF-4 spectrofluorometer. Special care was taken in order to obtain particle-free solutions.

The experiments were performed as follows: phosphofructokinase was diluted in a buffer containing 50 μM MNS to concentrations below 10^{-7} M and equilibrated for 2 hr. The samples were then mixed with the 0.5- to 8-fold volume of buffer, also containing 50 μM MNS. The increase of fluorescence was recorded for 10–15 min. Excitation wavelength used was 325 nm, emission wavelength was 420 nm. Unless otherwise stated, the experiments were performed in 0.1 M triethanolamine–HCl buffer containing 0.2 M KCl and 50 μM MNS (Buffer A).

3. Model and treatment of data

In previous studies, an open mode of association of phosphofructokinase represented as

$$2\,P_1 \rightleftarrows P_2 \rightleftarrows \frac{1}{2}\,P_3 \rightleftarrows \ldots\ldots \frac{1}{(n-1)}\,P_n$$

turned out to be sufficient for describing kinetic [2,3] and fluorescence [4] experiments. The species P_1 is regarded as an inactive subunit and P_2 is the smallest active entity of the enzyme. P_1 and P_2 are the predominant species if the concentration of the enzyme, c_T, is at least one order of magnitude lower than the dissociation constant, K_D, of the step $P_2 \rightleftarrows 2\,P_1$, assuming the dissociation constants of the other steps are of the same order of magnitude as K_D.

The binding of a given concentration of MNS to the particles P_1 and P_2 depends on the concentration c_{P_1} and c_{P_2} as well as on the number of binding sites and on the affinity of the fluorescence probe. At low concentrations of phosphofructokinase, the total MNS fluorescence F is given by

$$F = f_1 \cdot c_{P_1} + f_2 \cdot (c_T - \frac{1}{2}\,c_{P_1}) + f_3 \cdot c_{MNS\ (free)}$$

where f_1, f_2, and f_3 are the factors of proportionality of the fluorescence of MNS bound to P_1 and P_2 and of non-bound MNS respectively. When the fluorescence

is corrected for the low fluorescence of the non-bound probe, the concentration of P_1 can be calculated by the formula

$$c_{P_1} = (F - f_2 \cdot c_T) / (f_1 - f_2/2) \qquad \text{(eq. 1)}$$

inserting experimental values of f_1 and f_2 and expressing c_T in terms of the molecular weight of P_2.

The dissociation of P_2 into P_1 is concentration-dependent; after dilution of the enzyme, therefore, the fluorescence changes as c_{P_2} decreases and c_{P_1} increases. Using the equations introduced by Eigen and coworkers [8], the changes in c_{P_2} may be expressed by a concentration variable x,

$$x = x_o / [4 \cdot k_1 \cdot x_o \cdot (1 - e^{t/\tau}) + e^{t/\tau}] \qquad \text{(eq. 2)}$$

where x_0 is the value of x at time $t = 0$, and τ is the relaxation time given by

$$\tau = (k_2 + 4 k_1 \cdot c_{P_1}^{\infty})^{-1} \qquad \text{(eq. 3)}$$

where k_1 is the rate constant of association, k_2 is the rate constant of dissociation, and $c_{P_1}^{\infty}$ is the equilibrium concentration of P_1 at $t \to \infty$. If τ is known at different concentrations $c_{P_1}^{\infty}$, the rate constants and $K_D = k_2/k_1$ are easily derived from eq. 3. Though eq. 2 does not fit an exponential curve, estimations of τ could be derived directly from the recorder traces under experimental conditions. Using an estimation of k_1, the rate constants were improved by fitting eq. 2 to the experimental curves.

4. Results

Fluorescence factors of the phosphofructokinase MNS system. Phosphofructokinase diluted to low concentrations in the presence of fructose 1,6-bisphosphate or other positive effectors has optimum activity and shows a linear relationship between enzyme activity and enzyme concentration [1]. Assuming $c_{P_1} = 0$ under these conditions, f_2 is determined from the linear increase of MNS fluorescence at rising enzyme concentrations. On the other hand, phosphofructokinase is completely dissociated in the presence of 3 M urea, i.e. $c_{P_2} = 0$, thus allowing for the determination of f_1.

4.1. *Determination of rate and equilibrium constants*

It was confirmed by stopped-flow experiments that the fluorescence enhancement of MNS upon dilution of phosphofructokinase proceeds in a two-step manner. The first part of the process is very rapid and is completed within the mixing time of the instrument (2 msec). A second part of the process was slow at low enzyme concentrations and could be recorded without application of rapid mixing techniques. Fig.1 shows a reproduction of typical recorder traces together with the curve calculated to eq.2. Fig.2 is a plot of $1/\tau$ vs. c_{P_1} resulting from experiments at pH 7.9. The rate constants k_1 and k_2 were extrapolated from the slope of the straight line and the intercept on the ordinate respectively as $k_1 = 2.38 \times 10^4\ M^{-1}\ sec^{-1}$ and $k_2 = 1.11 \times 10^{-2}\ sec^{-1}$; K_D was 4.66×10^{-7} M.

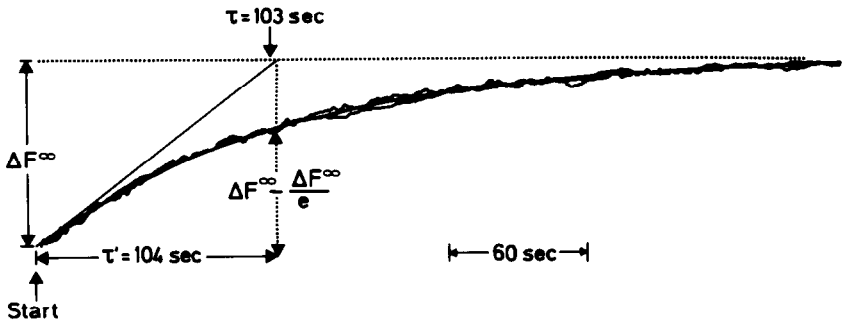

Fig.1. A typical record of fluorescence increase upon dilution of phosphofructokinase. At $t = 0$, a stock solution containing 0.01 mg phosphofructokinase/ml in buffer A (pH 7.6; 30°C) was diluted to 0.0039 mg/ml. The records of two experiments are superimposed. Although the curves do not fit an exponentail function, approximate values of τ (designated τ') are easily derived as shown in the figure. Improved values of τ were obtained by fitting eq. 2 to the experimental curves as shown by the smooth line (τ = 103 sec, $k_1 = 1.02 \times 10^4\ M^{-1}\ sec^{-1}$, and $x_0 = 1.37 \times 10^{-8}$ M).

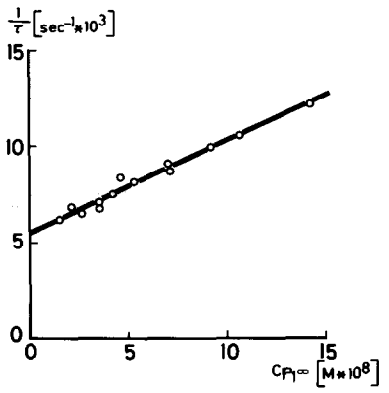

Fig.2. Plot of $1/\tau$ vs $c_{P_1}^{\infty}$ according to eq. 3. The experiments were performed at pH 7.9 and 20°C. the constants evaluated were $k_1 = 1.19 \times 10^4$ M^{-1} sec^{-1}, $k_2 = 5.55 \times 10^{-3}$ sec^{-1}, and $K_D = 4.66 \times 10^{-7}$ M.

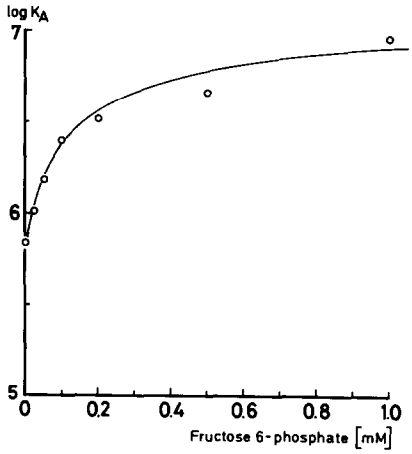

Fig.3. Dependence of the association constant K_A on fructose 6-phosphate concentration at pH 7.4 and 20°C.

4.2. *pH-Dependence of phosphofructokinase dissociation*

At 20°C, the dissociation constant K_D rises from 4.66×10^{-7} M at pH 7.9 to 1.05×10^{-6} M at pH 7.6, and to 1.8×10^{-5} M at pH 7.0. It may be noted that the presence of 0.2 M KCl favours association in these experiments. Measurements at pH 7 or in the absence of KCl were affected by rapid growth of turbidity of the enzyme solution.

4.3. *Dependence of phosphofructokinase dissociation on the presence of substrates and effectors*

The stabilization of specific activity of phosphofructokinase by its substrate fructose 6-phosphate or by allosteric effectors was reflected by the decrease of K_D of almost one order of magnitude from 1.4×10^{-6} M in the absence of substrate at pH 7.4 to 1.1×10^{-7} M in the presence of 1.0 mM fructose 6-phosphate. In fig.3, the logarithms of the association constants, K_A, were plotted against fructose 6-phosphate concentration, demonstrating a hyperbolic saturation behaviour for the substrate effect on association. Half-maximum association occurs at 0.1 mM fructose 6-phosphate; this concentration corresponds well with the Michaelis constant of the enzyme at low ATP concentrations. A much more pronounced associating effect was observed in the presence of the allosteric activator fructose 1,6-bisphosphate. The K_D in the presence of 0.5 mM fructose 1,6-bisphosphate was in the range of 10^{-8} M. It must be noted, however, that the sensitivity of the fluorescence method should be improved in order to obtain kinetic parameters under conditions which strongly favour association.

5. Discussion

The strong fluorescence of phosphofructosekinase-bound MNS in combination with the preferential binding of the probe to the inactive subunits of the enzyme [4] permits the determination of dissociation parameters at low enzyme concentration. At these enzyme levels, observation is limited to a single dissociation step as confirmed by the agreement of the experimental and calculated course of fluorescence upon dilution.

The mean phosphofructokinase content of rabbit muscle is about 10^{-6} M. This suggests that at low substrate concentrations as present in the resting muscle and at physiological pH, phosphofructokinase is at least partially present in an inactive dissociated form. Rising concentrations of substrate and fructose 1,6-bisphosphate decrease the dissociation constant by orders of magnitude, thus leading to an increase in the amount of active enzyme in addition to the allosteric effects exerted by these substrates. It follows from the rate constants of association that under cellular conditions the half-time of the molecular weight transitions is between 10 and 30 sec.

This is similar to the time requirement of interconversion reactions [9] and suggests that the association-dependent changes of the concentration of active phosphofructokinase may be responsible for establishing a certain functional state of this regulatory enzyme.

Acknowledgements

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