Binding of ATP to nucleoside-diphosphate kinase: a kinetic study

Ioan Lascu, Elena Presecan and Ioan Proinov

Department of Biochemistry, Medical and Pharmaceutical Institute, Pasteur Street 6, R-3400 Cluj-Napoca, Romania

Received 28 April 1986

The binding of nucleotides to pig heart nucleoside-diphosphate kinase was studied using Rose Bengal as an optical probe. ATP, in the absence of Mg^{2+}, binds slowly to the enzyme, with a second order rate constant of about 3000 $M^{-1}\cdot s^{-1}$, whereas in its presence the binding is much faster. This finding suggests the regulation of the nucleoside-diphosphate kinase activity by uncomplexed ATP, and that ATP binds normally to the enzyme via a metal ion bridge.

Nucleoside-diphosphate kinase *Rose Bengal* *Substrate binding* *Metal ion bridge* *Conformational change* *ATP-Mg^{2+} complex*

1. INTRODUCTION

The nucleoside-diphosphate (NDP) kinases from various origins show remarkably uniform properties, which suggest a highly conservative evolution [1,2]. The mechanism is ping-pong, and in all cases studied the phosphate group was transiently bound by covalent linkage to a histidine side chain [1,3]. This enzyme is a very active one, the value of k_{cal}/K_m being about 10^8 $M^{-1}\cdot s^{-1}$, i.e. in the class of diffusion-controlled enzyme reaction rates [4].

Here we used the dye Rose Bengal as an optical probe with the aim to elucidate the binding pattern of substrates to NDP kinase. This approach allows one to obtain information on the equilibrium as well as on the kinetics of the binding by competition of the dye with the substrates.

2. MATERIALS AND METHODS

The nucleotides were obtained from Boehringer, Mannheim. NDP kinase was purified from pig hearts by affinity chromatography on Blue Sepharose as described [5]. The molar enzyme concentration was expressed as subunit concentration assuming an M_r of 17000 and $A^{1\%}_{280nm}$ equal to 12.6 [6]. The ratio A_{280}/A_{260} was always 1.70 ± 0.02, which indicates no tightly bound nucleotides. Rose Bengal was purified by preparative thin-layer chromatography on silica gel plates with 2-butanol as eluent. The concentration of the dye was calculated using an absorption coefficient of 95 $mM^{-1}\cdot cm^{-1}$ at 545 nm [7]. The buffer used in this study was 20 mM sodium phosphate (pH 6.9) containing 1.0 mM EDTA. Difference absorption spectra and difference absorbances at fixed wavelengths were obtained using a Specord UV-VIS recording spectrophotometer (Zeiss, Jena, GDR). The temperature was kept constant at 25°C. By titration of the dye with a large molar excess of enzyme and extrapolation to infinite enzyme concentration, the difference absorption coefficient was obtained.

3. RESULTS

3.1. *Equilibrium studies*

Rose Bengal was a useful probe for NDP kinase since the difference between the spectra of the dye in the presence and absence of the enzyme had an isosbestic point, which indicated a single mode of binding. The $\Delta\epsilon_{mM}$ had the large value of 50 ± 2 $mM^{-1}\cdot cm^{-1}$ at 560 nm. Moreover, this value

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

was not changed by the presence of ADP or ATP. This means that the variation in the measured ΔA by adding nucleotides was due to the displacement of the bound dye and not to a variation of the $\Delta\epsilon$. By Scatchard analysis it was shown that one dye molecule bound per enzyme subunit, with a K_d of 0.8–1.3 μM. To ascertain if the dye bound to the active site, competition experiments with nucleotides were performed (fig.1). By adding ADP to the enzyme-dye complex there was a decrease in the difference absorbance. By quantitative analysis it was found that ADP bound to the enzyme weakly (K_d = 200–400 μM) in a competitive mode. AMP bound poorly to the enzyme. ATP at fairly low concentrations produced a sharp decrease in the bound dye concentration by about 50%, but the increase in the ATP concentration up to several millimolar failed to decrease this value further. A similar picture was obtained when using excess enzyme over the dye, which excluded a

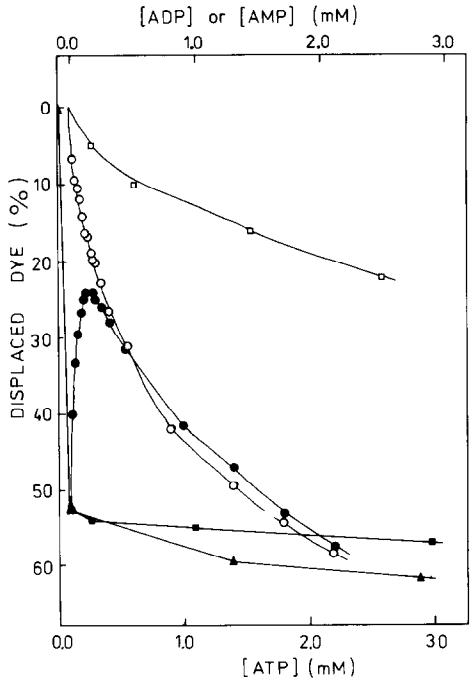

Fig.1. Effect of nucleotides on the binding of Rose Bengal to NDP kinase. The reference cell contained 20 μM Rose Bengal, whereas the sample cell contained 20 μM dye, 4.63 μM NDP kinase, and the indicated concentration of ATP (■), ADP (○), AMP (□), 93 μM ATP + ADP (●), and 93 μM ATP + AMP (▲); final volume, 1.0 ml; 0.5 cm optical path.

cooperative effect of the two ligands. The behaviour of ATP was typical for noncompetitive binding. Its binding to the enzyme produced the displacement of the dye by decreasing its affinity for the enzyme-ATP complex, and not by direct competition. It seems therefore that ADP and ATP bound to separate sites. This was further suggested by the effect of ADP in the presence of ATP. A detailed analysis in support to this view will be reported elsewhere.

3.2. *Kinetic studies*

A further difference between the behaviour of ADP and ATP as competitors with the dye was the rate of the decrease of the difference absorbance when the nucleotide was added to the enzyme-dye complex. In the former case the equilibrium was attained within the mixing time (1 s or less), whereas in the latter case several minutes were necessary in order to obtain a stable reading of the difference absorbance. A very similar picture was previously obtained using Cibacron blue 3G-A as an optical probe [6]. The decrease in the difference absorbance followed a first order law. The measured rate constant was proportional to the concentration of ATP despite the large molar excess of ATP over the dye. Therefore the rate-limiting step was not the dissociation of the dye from its complex with the enzyme [8]. Since the treatment of consecutive reversible reactions is complicated and sometimes ambiguous, we chose a new experimental set-up, which allowed a clear distinction between the binding patterns by simple inspection. As shown in fig.2, instead of adding the nucleotide to the enzyme-dye complex, the enzyme was added to the nucleotide-dye mixture. It was noted that the difference absorbance quickly approached the value obtained in the absence of nucleotide, and slowly decreased to the equilibrium value. The measured first order rate constant of the slow reaction was equal irrespective of the order of mixing of the reagents, which indicated that the rate-limiting event was the same. If, however, the dye was added to the enzyme-ATP complex, the difference absorbance attained equilibrium within the mixing time. These results suggest that ATP bound to the enzyme much more slowly than Rose Bengal, so that in the early phase of the reaction the dye bound just as much as in the absence of ATP. At longer reaction times, some of

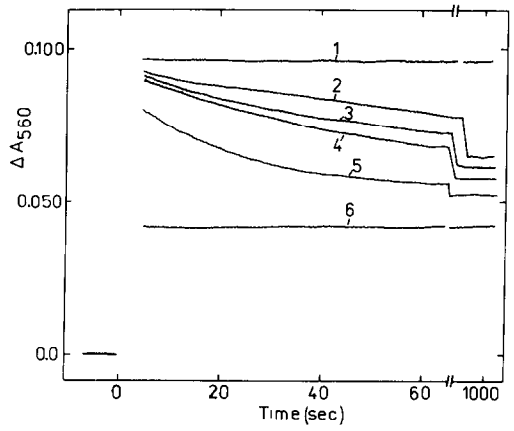

Fig.2. Kinetics of change in the difference absorbance upon addition of NDP kinase to a Rose Bengal-ATP mixture. Both cells contained 20 μM dye and ATP at the final concentration 0.0 (trace 1), 61 (trace 2), 91.3 (trace 3), 122 (trace 4), 243 (trace 5), and 550 μM ATP plus 6 mM $MgCl_2$ (trace 6); final volume, 1.8 ml; 1.0 cm optical path. At zero time NDP kinase at a final concentration of 3.5 μM was added to the sample cell and rapidly mixed.

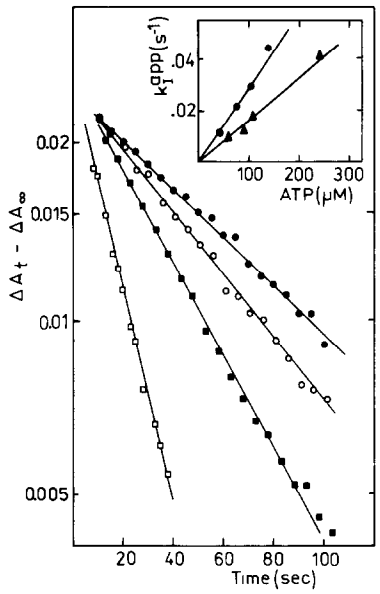

Fig.3. Semilog plot of the data taken from traces 2–5 from fig.2 (respectively, ●,○,(■ and □). Inset: plot of the apparent first order rate constant k_1^{app} vs the concentration of ATP. The concentration of Rose Bengal was 10 μM (●) and 20 μM (▲).

the dye dissociated from the complex due to the reduced affinity of the dye for the enzyme-ATP complex.

The decrease of the difference absorbance followed a first order kinetics (fig.3). The measured rate constant k_1^{app} was proportional to the concentration of ATP, and the slope of the plot was proportional to the reciprocal of the dye concentration. This suggests that under this particular set of conditions the measured first order rate constant (k_1^{app}) describing the dissociation kinetics by the effect of a competitor [8] reduced to:

$$k_1^{app} = \frac{k_A[A]}{K_d[D]} \quad (1)$$

where k_A represents the second order rate constant of the binding reaction of ATP to the enzyme and K_d denotes the dissociation constant of the enzyme-dye complex. k_A was found to be equal to 3000 and 3300 $M^{-1} \cdot s^{-1}$ (two experiments), i.e. 10^3–10^4-times smaller than a typical rate constant [9]. This is indicative of a conformational change induced by ATP. When the experiments were performed in the presence of Mg^{2+}, only a rapid reaction was noted, which indicates rapid binding of MgATP to NDP kinase.

Similar results were obtained with GTP, therefore the triphosphate chain is responsible for the differential behaviour in the presence and absence of Mg^{2+}.

4. DISCUSSION

Several lines of evidence suggest that NDP kinase exists in two or more conformations, with possible relevance for the catalysis [1,10]. The above kinetic evidence is particularly important because free ATP is not only a potent inhibitor, but also because of the slow binding to NDP kinase, the enzyme would have hysteretic properties which may severely impair the catalytic efficiency. Probably several of the previous steady-state kinetic experiments should be revised, particularly when Mg^{2+} was not in large excess. This finding also suggests a way of regulation of NDP kinase activity in the living cell, by strong inhibition with free ATP. This type of regulation may be

significantly related to the discontinuous synthesis of DNA.

The results presented demonstrate the very stringent requirement of NDP kinase for the correct conformation of the triphosphate chain. The differential effect of ATP in the presence and absence of Mg^{2+} strongly suggests that ATP binds to NDP kinase via a metal ion bridge for catalysis.

REFERENCES

[1] Parks, R.E. and Agarwal, R.P. (1973) in: The Enzymes (Boyer, P.D. ed.) vol.8, part A, pp.307–333, Academic Press, New York.
[2] Robinson, J.B., Brems, D.N. and Stellwagen, E. (1981) J. Biol. Chem. 256, 10769–10773.
[3] Colomb, M.G., Chéruy, A. and Vignais, P.V. (1972) Biochemistry 8, 3378–3386.
[4] Davies, J.R. and Scopes, R.K. (1981) Anal. Biochem. 114, 19–27.
[5] Lascu, I., Duc, M. and Cristea, A. (1981) Anal. Biochem. 113, 207–211.
[6] Lascu, I., Pop, R.D., Porumb, H., Presecan, E. and Proinov, I. (1983) Eur. J. Biochem. 135, 497–503.
[7] Wu, C.-W. and Wu, F.Y.-H. (1973) Biochemistry 12, 4349–4355.
[8] Gutfreund, H. (1974) in: Chemistry of Macromolecules (Gutfreund, H. ed.) pp.261–286, Butterworths, London.
[9] Hammes, G.G. and Schimmel, P.R. (1970) in: The Enzymes (Boyer, P.D. ed.) vol.2, pp.67–114, Academic Press, New York.
[10] Duc, M., Lascu, I., Porumb, H., Gozia, O., Schell, H.D. and Bârzu, O. (1981) FEBS Lett. 127, 281–284.