THE INTERACTION OF NUCLEOTIDES WITH KINASES, MONITORED BY CHANGES IN PROTEIN FLUORESCENCE

N.C. PRICE

Johnson Research Foundation, University of Pennsylvania, School of Medicine, Philadelphia, Pa. 19104, USA

Received 22 May 1972

1. Introduction

Kinases, enzymes which catalyze the transfer of a phosphoryl group of a triphosphate nucleotide, require a divalent metal ion for activity [1]. The fact that the paramagnetic Mn^{2+} ion can substitute for Mg^{2+} has allowed considerable insight into the mechanism of action and conformational properties of these enzymes to be gained [2, 3]. A knowledge of the dissociation constants of the nucleotide substrates from the enzyme (in the absence of divalent metal ions) facilitates analysis of the binding data in the presence of divalent metals [4] and of the kinetics of the enzymatic reaction. This paper describes a rapid method for the estimation of these dissociation constants, based on changes in the protein fluorescence on addition of the nucleotides. The technique has been applied to creatine kinase (CPK) and pyruvate kinase (PK), both from rabbit muscle, and the results are discussed in the light of the known properties of these enzymes.

2. Materials and methods

CPK and PK were isolated from frozen rabbit muscle (Pel-Freeze) according to the previously published procedures [5, 6]. Glycyl-L-tryptophan and triethanolamine hydrochloride were purchased from Sigma, *N*-2-hydroxyethyl piperazine-*N'*-2-ethane sulphonic acid (HEPES) from Calbiochem, and the nucleotides from Boehringer. Fluorescence measurements were made on a Perkin Elmer MPF2A fluorescence spectrophotometer, equipped with a thermostatted cell compartment. Fluorescence titrations were performed essentially as described in a previous paper [7] in which the binding of NAD^+ to rabbit muscle glyceraldehyde-3-phosphate dehydrogenase was studied. Equal aliquots of concentrated solutions of the nucleotides were added to two cuvettes, one containing a solution of the kinase, the other containing a solution of glycyl–tryptophan of the same absorbance (normally about 0.15) at the excitation wavelength, 295 nm. Fluorescence emission was measured at 350 nm. The glycyl–tryptophan solution was used as a blank to correct for the absorption of some of the excitation radiation by the added nucleotide (the "inner filter" effect) and also for dilution effects. In a typical titration each of these effects contributed about a 6% decrease in the fluorescence of the blank (at a nucleotide concentration of 3 mM).

3. Results and discussion

After correction for the "inner filter" and dilution effects of the added nucleotide, the effect of ADP on the fluorescence of PK is shown in fig. 1, where the variability of the readings in three separate titrations is indicated. The data is plotted in double reciprocal form in fig. 2, from which the values of the limiting protein fluorescence (at saturating concentrations of ADP) and the dissociation constant for the ADP–PK complex can be obtained. Since in these experiments the concentration of enzyme (typically 15 μM in sites) is much lower than the nucleotide concentrations, we are justified in setting the free nucleotide concentration equal to the total nucleotide concentration for these plots.

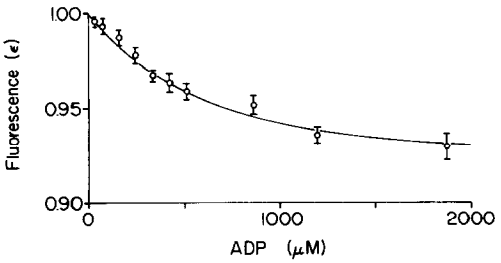

Fig. 1. Relative fluorescence (ε) of PK on addition of ADP. Conditions 0.05 M K^+-HEPES, pH 7.5 (0.075 M KCl added) T = 22°. (o): experimental points with variation in 3 titrations. The solid line is calculated assuming parameters shown in the table.

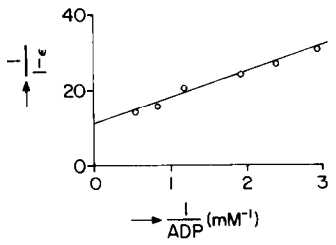

Fig. 2. Double reciprocal plot of data in fig. 1. (o): average points of 3 titrations. The straight line is computed on a least square basis.

The values of the limiting fluorescence and dissociation constants obtained by this procedure are summarized in table 1. No evidence was found for any cooperative interactions (either positive or negative) between the nucleotide sites in either enzyme. It is seen that the effects on the protein fluorescence are typically between 5 and 20% quenching in these cases. Although these effects are small, the measurements are reasonably reproducible as indicated in the table and figures. The results in the table represent the average of three titrations.

It is possible that the quenching arises from direct interaction of the nucleotides with the amino acid residues (primarily tryptophan [8]) responsible for the fluorescence of the enzymes. Alternatively, the quenching could reflect some nucleotide-induced conformational changes in the enzymes, which lead to alterations in the environment of the tryptophan residues. Evidence for nucleotide-induced conformational changes in CPK has been obtained from studies of temperature-jump relaxation [9], sulphydryl group reactivity [10], and of enzyme derivatives labelled at the "essential" sulfhydryl group with either a spin label [11] or a fluorescent label [12]. Less evidence is available on the effects of nucleotides on PK, but ADP has been shown to protect PK against inactivation by mercuribenzoate [13] and trinitrobenzene

Table 1
Dissociation constants and limiting fluorescence values for the nucleotide complexes of CPK and PK.

Enzyme	Nucleotide	Dissociation constant (μM)	Limiting fluorescence *	Conditions
CPK	ADP	230 ± 20	0.896 ± 0.002	0.1 M Triethanolamine, pH 7.6 (30°)
CPK	ADP	95 ± 10	0.905 ± 0.002	0.05 M K^+-HEPES, pH 8.0 (23°)
CPK	ATP	600 ± 200	0.953 ± 0.01	As above
PK	ADP	625 ± 100	0.909 ± 0.01	0.05 M K^+-HEPES + 0.075 M KCl, pH 7.5 (22°)
PK	ATP	1250 ± 400	0.930 ± 0.01	As above
PK	ADP	1170 ± 100	0.846 ± 0.012	0.05 M TMA^+-HEPES + 0.075 M TMACl pH 7.5 (22°)
PK	ATP	1950 ± 200	0.815 ± 0.02	As above

* Relative to fluorescence before addition of nucleotide = 1.000

sulphonic acid [14], which may be a result of ADP-induced conformational changes in the enzyme. In the experiments reported here, no significant shift of the emission maximum of the enzyme fluorescence was observed on addition of the nucleotides.

The dissociation constant of ADP from CPK was measured under two different sets of conditions (see table). An estimate of this value under the second set of conditions (0.05 M K^+-HEPES, pH 8.0) is available from the computer analysis of the equilibria in the CPK–Mn^{2+}–ADP system [4]. The value for the dissociation constant of ADP from CPK by this procedure was 110 μM, in good agreement with the value reported here.

ATP binds much more weakly to CPK (see table), as had been previously found [15] (though under different conditions). The uncertainty in the estimate of this dissociation constant is rather larger than for ADP, since the effect of ATP on the fluorescence of CPK is smaller.

In the case of PK, the dissociation constants are larger than for CPK and depend on the ionic composition of the solution. The activating cation, K^+, strengthens the binding of the nucleotides compared with the nonactivating tetramethylammonium ion (TMA^+). Direct binding studies of the nucleotides to PK have not been reported. Mildvan and Cohn [13] using a kinetic protection method report a value of 140 μM for the ADP–PK dissociation constant (in the presence of K^+) under slightly different conditions; however their conclusion is complicated by the finding of a second type of ADP site with a 20-fold higher dissociation constant.

0.1 M K^+ quenches the fluorescence of PK (relative to TMA^+) by about 13%, an effect which can be ascribed to the K^+-induced conformational change in the enzyme [16]. The results in the table can be rationalised by postulating that PK exists in two conformational states, one of which (with a lower fluorescence) preferentially binds the nucleotides. Addition of K^+ displaces the equilibrium towards this form, whereas in the presence of TMA^+ the equilibrium lies further to the side of the other form (of higher fluorescence). Since addition of Mg^{2+} in the presence of K^+ further quenches the fluorescence of PK (by about 6%), it would be expected that the fluorescence quenching by added nucleotides in the presence of both K^+ and Mg^{2+} would be smaller than in the presence of K^+ alone. This expectation has been borne out in preliminary experiments.

The protein fluorescence technique thus provides a useful method for the measurement of dissociation constants for the nucleotide/kinase complexes. It should also be applicable to the study of those kinases which do not contain tryptophan residues (e.g. porcine muscle adenylate kinase). However in such cases, the weaker fluorescence arising from tyrosine residues at excitation wavelengths where the "inner filter" effects of the added nucleotides are relatively small can give rise to poorer reproducibility of the measurements.

Acknowledgements

I wish to thank Miss Hannelore Diefenback for help with the enzyme preparations and Drs. George Reed and Fred Kayne for helpful discussions.

References

[1] R. Nordlie and H.A. Lardy, in: The Enzymes, Vol. 6, eds. P.D. Boyer, H.A. Lardy and K. Myrbäck (Academic Press, New York, 1962) p. 36.
[2] M. Cohn, J.S. Leigh, Jr. and G.H. Reed, Cold Spring Harbor Symp. Quant. Biol. XXXVI (1971) 533.
[3] M. Cohn and J. Reuben, Accounts Chem. Res. 4 (1971) 214.
[4] G.H. Reed, M. Cohn and W.J. O'Sullivan, J. Biol. Chem. 245 (1970) 6547.
[5] S.A. Kuby, L. Noda and H.A. Lardy, J. Biol. Chem. 209 (1954) 191.
[6] A. Tietz and S. Ochoa, Arch. Biochem. Biophys. 78 (1958) 477.
[7] N.C. Price and G.K. Radda, Biochim. Biophys. Acta 235 (1971) 27.
[8] L. Brand and B. Witholt, in: Methods in Enzymology, Vol. 11, ed. C.H.W. Hirs (Academic Press, New York, 1967) p. 830.
[9] G.G. Hammes and J.K. Hurst, Biochemistry 8 (1969) 1083.
[10] W.J. O'Sullivan and M. Cohn, J. Biol. Chem. 241 (1966) 3116.
[11] J.S. Taylor, A. McLaughlin and M. Cohn, J. Biol. Chem. 246 (1971) 6029.
[12] N.C. Price, unpublished results.
[13] A.S. Mildvan and M. Cohn, J. Biol. Chem. 241 (1966) 1178.
[14] P.F. Hollenberg, M. Flashner and M.J. Coon, J. Biol. Chem. 246 (1971) 946.
[15] S.A. Kuby, T.A. Mahowald and E.A. Noltmann, Biochemistry 1 (1962) 748.
[16] C.H. Suelter, R. Singleton, Jr., F.J. Kayne, S. Arrington, J. Glass and A.S. Mildvan, Biochemistry 5 (1966) 131.