Biochimica et Biophysica Acta, 370 (1974) 153–159

BBA 67336

STUDIES ON MANGANESE–SUBSTRATE COMPLEXES OF ARGININE KINASE FROM *PANULIRUS LONGIPES*

W. J. O'SULLIVAN[a], E. SMITH[b], B. E. CHAPMAN[a] and K. H. MARSDEN[c]

[a] *Department of Medicine, University of Sydney, Sydney, N.S.W., 2006,* [b] *Department of Biochemistry, Australian National University, Canberra, A.C.T., 2600 and* [c] *Department of Physics, University of New South Wales, Kensington, N.S.W., 2033 (Australia)*

(Received April 29th, 1974)

SUMMARY

Measurements of the relaxation rate of water protons in the presence of Mn^{2+} were used to demonstrate the formation of a ternary MnADP–enzyme complex with arginine kinase (EC 2.7.3.3) from *Panulirus longipes*. The enhancement of the proton relaxation rate of the ternary complex was determined to be 18 and the dissociation constant of $MnADP^-$ from the complex as 30 μM. An independent estimate of the dissociation constant for $MnADP^-$ of 20 μM, was obtained by initial velocity measurements of the enzymic reaction.

Evidence for the formation of ternary complexes with the enzyme was also obtained for the manganous complexes of deoxy ADP, IDP, GDP, ATP, deoxy ATP and ITP. The enhancement of the proton relaxation rate was significantly decreased on the addition of L-arginine to MnADP–enzyme, or Mn–deoxy ADP–enzyme, indicative of the formation of a quaternary dead-end complex, enzyme–metal–substrate–L-arginine.

INTRODUCTION

Arginine kinase (ATP: L-arginine phosphotransferase, EC 2.7.3.3) catalyses the reversible phosphorylation of L-arginine by the reaction:

$$\text{L-arginine} + MATP^{2-} \rightleftharpoons \text{phosphoarginine} + MADP^-$$

The enzyme from various sources has been the subject of a number of kinetic and thermodynamic investigations [1]. In particular, detailed kinetic studies, with Mg^{2+} as the essential metal ion, on the enzyme from the West Australian crayfish, *Panulirus longipes* indicated that the reaction mechanism was of the rapid equilibrium random type [2, 3], though earlier studies with the enzyme from *Jasus verreauxi* had provided evidence for a ping-pong mechanism [4].

Complementary to these studies, magnetic resonance and kinetic studies, with Mn^{2+} as the activating metal ion, on enzyme from the former source were initiated. In particular, changes in the proton relaxation rate of water in the presence of Mn^{2+}

could be used to obtain an independent assessment of the interaction of various substrates with the enzyme [5, 6] and thus provide confirmatory evidence for the mechanism of the enzyme reaction.

Subsequently, a detailed study of the interaction of $MnADP^-$, in the presence and absence of L-arginine and various anions, with arginine kinase from *Homarus americanus* has been carried out by Buttlaire and Cohn [7, 8]. As some of the results with the *P. longipes* enzyme differ from, or supplement the findings of Buttlaire and Cohn [7, 8], they are briefly presented here.

EXPERIMENTAL PROCEDURE

Arginine kinase was prepared as described by Smith and Morrison [2]. It was dialysed exhaustively against 0.01 M *N*-ethylmorpholine·HCl, pH 8.0, before carrying out the magnetic resonance experiments, to remove traces of EDTA used in the preparation. Other materials were as described previously [2, 3]. Measurements of the proton relaxation rate of water were made with a 180–90°-pulsed nuclear magnetic resonance spectrometer, operating at 30 Mcycles/s [9]. Experiments were carried out with 0.12 mM $MnCl_2$, in 0.05 M *N*-ethylmorpholine·HCl, pH 8.0, at 20 °C.

The determination of the enhancement, ε, of the proton relaxation rate for a solution containing Mn^{2+} in the presence of a complexing species has been described previously [5, 6, 10]. Estimates of ε_t, the enhancement of the ternary enzyme–metal–substrate complex and of K_2 and K_s, the dissociation constants for the equilibria between enzyme and metal–substrate and enzyme and uncomplexed substrate, respectively, were obtained using the computer programme described by Reed et al. [11]. Characteristic enhancements for enzyme–metal and metal–substrate are denoted as ε_b and ε_a, respectively, and the dissociation constants of these complexes as K_D and K_I [10].

Initial velocity studies, with Mn^{2+} as the activating metal ion were carried out as described previously with Mg^{2+} [2].

RESULTS AND DISCUSSION

Binary and ternary complexes

Relatively small enhancements were seen with Mn^{2+} and enzyme alone as found for arginine kinase from other sources [7, 12]. It was not possible to obtain a unique solution to the proton relaxation rate data though extrapolation of the results indicated one metal-binding site per enzyme molecule with a value of ε_b, the enhancement of the enzyme–metal complex, as approx. 9 with a dissociation constant of approx. 1 mM. These values are in substantial agreement with those of Buttlaire and Cohn [7], viz. ε_b, 9.6; K_D, 0.55 mM for the *H. americanus* enzyme. The observed enhancements were substantially reduced in the presence of 0.25 M KCl, indicating that the direct binding of Mn^{2+} to the enzyme is probably non-specific.

A large increase in enhancement was observed when arginine kinase was titrated with ADP in the presence of $MnCl_2$. The results were qualitatively similar to those obtained with the enzyme from *Homarus vulgaris* and *H. americanus* [12, 7] and indicated that the enzyme was of the Class I type of Cohn, with $\varepsilon_t > \varepsilon_b$ [5]; i.e. the most probably coordination scheme for the metal ion in the ternary complex

involves a nucleotide bridge to the enzyme. Analysis of the data, (assuming one nucleotide-binding site per molecule of enzyme and a molecular weight of 37 000 [1]) indicated a best fit with the values, $K_2 = 30\ \mu M$, $K_s = 82\ \mu M$ and $\varepsilon_t = 18.0$ with a percentage standard deviation of 7% (Fig. 1).

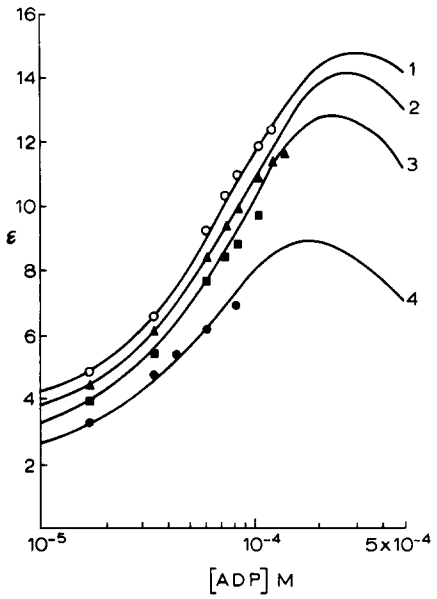

Fig. 1. Proton relaxation rate titration data for arginine kinase and $MnCl_2$ (0.12 mM) with ADP. Concentrations of arginine kinase are: ○—○, line 1, 0.44 mM; ▲—▲, line 2, 0.35 mM; ■—■, line 3, 0.26 mM; ●—●, line 4, 0.12 mM. Experiments were carried out in 0.05 M *N*-ethylmorpholine–HCl, pH 8.0, at 20 °C. Solid curves are drawn with $K_D = 1$ mM, $\varepsilon_b = 9.0$, $K_1 = 0.03$ mM, $\varepsilon_a = 1.7$, $K_2 = 30\ \mu M$, $\varepsilon_t = 18.0$, $K_s = 82\ \mu M$. Values of K_2, K_s and ε_t taken from minimum percentage of standard deviation (7.0) in regression analysis.

These values would indicate that the metal–nucleotide binds approx. three times more tightly to the enzyme than does ADP^{3-}, a result very similar to the 2-fold difference observed with creatine kinase [9, 11]. They are substantially similar to those of Buttlaire and Cohn [7] on the *H. americanus* enzyme, though these workers determined a somewhat tighter binding constant (8 μM) for $MnADP^-$ (see Table I). It should be noted that our experiments were carried out over a narrower range of ADP concentration (17–160 μM compared to approx. 0.015–2 mM in the experiments of Buttlaire and Cohn [7]) which could account for the discrepancy in the results and the relatively high standard deviation [10]. However, the results could equally well reflect minor differences in the enzyme from two sources as our results are in good agreement with the kinetically determined dissociation constant of 20 μM (see below). Further, it should be noted that kinetic experiments with the *H. vulgaris* enzyme indicated a dissociation constant of approx. 30 μM for the interaction of MnADP with free enzyme (see Table II of ref. 12).

When tested under similar conditions, deoxy ADP gave enhancement values about 75% of those observed with ADP (see Fig. 2A). IDP gave smaller values still

TABLE I

SUMMARY OF CONSTANTS FOR MnADP–ENZYME INTERACTION

Proton relaxation rate results on the *P. longipes* enzyme are compared to the results of Buttlaire and Cohn [7] on the *H. vulgaris* enzyme (in parentheses) and to the kinetically determined value for the dissociation constant of $MnADP^-$ from the enzyme–metal–substrate complex.

Constant	Proton relaxation rate measurements	Kinetic measurements
K_2	30 μM (8 μM)	20 ($\pm$9) μM
K_s	82 μM (50 μM)	—
ε_t	18.0 (18.9)	—

and no significant effect was seen with GDP. Experimentally, the values corresponded to ADP > deoxy ADP > IDP > GDP, which is the same order as was observed for the maximal velocities of the magnesium complexes of the nucleotides [3] in kinetic experiments. However, no conclusions about any correlation between these factors can be drawn in the absence of accurate information on the proton relaxation rate parameters. For nucleoside triphosphates, the formation of ternary complexes could be inferred with experimental enhancements in the rank order ATP > deoxy ATP > ITP. (Graphical analysis [13] of the data with ATP indicated an ε_t value of

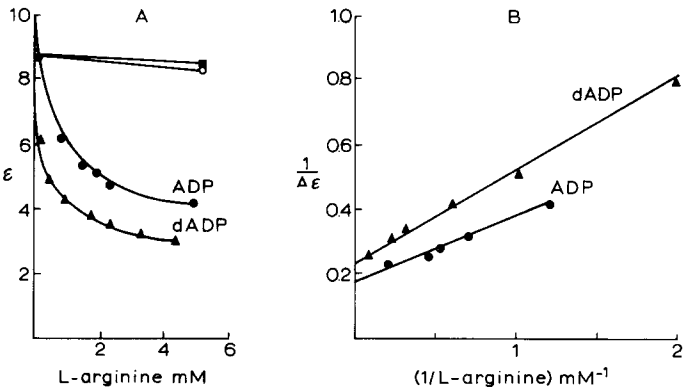

Fig. 2. (A) Titration of ternary MnADP–enzyme (●—●) and Mn–deoxy ADP–enzyme complexes (▲—▲) with L-arginine. Solutions contained ADP or deoxy ADP (0.11 mM), $MnCl_2$ (0.12 mM) and arginine kinase (0.19 mM) in addition to the indicated concentrations of L-arginine. The effects of 5 mM D-arginine (○) and 5 mM creatine (■) are also shown. Other conditions as for Fig. 1. (B) Double reciprocal plot of the change in enhancement as a function of L-arginine concentration for MnADP–enzyme (●—●) and Mn –deoxy ADP–enzyme (▲—▲).

15 and K_2 equal to 300 μM, compared to values of ε_t, 17 and K_2, 80 μM obtained by Buttlaire and Cohn [7]. A slight increase in enhancement with time was also observed with the MnATP–enzyme complex, possibly indicative of a weak ATPase activity [12, 13]). No significant changes were seen with the nucleotides UDP, CDP, 5′-adenosine methylene diphosphonate, CTP or UTP.

The reaction of arginine kinase with dithiobisnitrobenzoic acid under the same experimental conditions as used for the magnetic resonance studies, resulted in the

loss of the nucleotide binding site, as judged by proton relaxation rate measurements. A similar effect has been observed on reaction of dithiobisnitrobenzoic acid with rabbit muscle pyruvate kinase [19]. Using a value of $1.36 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ for the extinction coefficient of the nitrothiophenolate ion at 412 nm [14], it was estimated that approx. three sulphydryl groups had been reacted in this experiment.

Formation of enzyme–MnADP–guanidine complexes

The formation of a complex with L-arginine and the enzyme–MnADP complex was deduced from the decrease in the proton relaxation rate when arginine (5 mM) was added to a solution containing $MnCl_2$ (0.12 mM), ADP (0.11 mM) and arginine kinase (0.19 mM active sites). The formation of such a complex had been deduced previously from kinetic studies [2]. Decreasing the temperature from 20 to 0 °C caused a marked decrease in the proton relaxation rate for the quaternary complex but had only a slight effect on the ternary complex. The results were qualitatively similar to those observed previously for arginine kinase (see, in particular, Fig. 5 of ref. 12 and Table I of ref. 7) and also to observations on the formation of the quaternary MnADP–creatine kinase-creatine complex [15, 17]. They provide substantial evidence for the formation of the dead-end complex, MnADP–enzyme–L-arginine and thus the contention that the enzyme reaction proceeds via a rapid random equilibrium mechanism rather than a ping-pong mechanism [2–4].

The "L-arginine effect" could be titrated (Fig. 2A) and showed saturation behaviour. The concentration of L-arginine at the mid-point of this saturation curve was 1 mM, which would correspond to the dissociation constant for the interaction of L-arginine with the enzyme–MnADP complex (Fig. 2B) [17]. Though not a true dissociation constant for the interaction of L-arginine with the enzyme–MnADP complex, as the enzyme was not completely saturated with MnADP, it is in good agreement with the kinetically obtained value of 0.81 mM for the dissociation constant (K_i) reported previously for the interaction of L-arginine with the MgADP–enzyme complex [2].

Both D-arginine and creatine, which are weak competitive inhibitors with respect to arginine [3, 7] were used as controls for the demonstration of the formation of an enzyme–MnADP–L-arginine complex. Creatine (5 mM) and D-arginine (5 mM) both caused slight decreases in enhancement (see Fig. 2A) when added to solutions containing the same concentrations of $MnCl_2$, ADP and enzyme, indicating that the effect observed with L-arginine was not non-specific.

A similar, though smaller effect of L-arginine with Mn–deoxy ADP–enzyme also illustrated in Fig. 2. Analysis of the saturation effect (Fig. 2B) indicated a dissociation constant of 0.9 mM for L-arginine from the Mn–deoxy ADP–enzyme–L-arginine complex.

Kinetic studies of the reverse reaction of arginine kinase with $MnADP^-$ as a substrate

Initial velocity studies with $MnADP^-$ as a substrate of the reverse reaction [2] were carried out as described previously for $MgADP^-$. The initial velocity pattern obtained is shown in Fig. 3 and, by computer fitting of the data to the SEQUEN programme [18] the dissociation constant for the interaction of $MnADP^-$ with the enzyme, equivalent to K_2 from the proton relaxation rate experiment was determined as $20(\pm 9)\ \mu M$. These values are calculated on the assumption that the mechanism

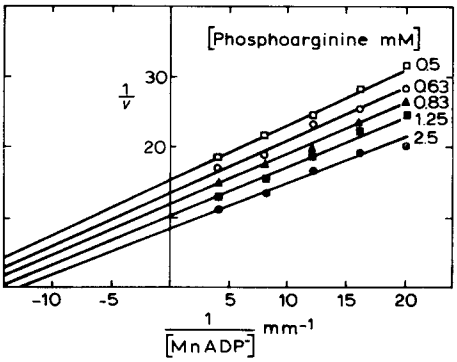

Fig. 3. Initial velocity pattern obtained with $MnADP^-$ as the variable substrate and phosphoarginine at a number of fixed concentrations. Velocities are expressed as μmoles/min per μg of arginine kinase. Concentrations of phosphoarginine are: □—□, 0.5 mM; ○—○, 0.625 mM; ▲—▲, 0.83 mM; ■—■, 1.25 mM; ●—●, 2.5 mM. Experiments were carried out in 0.05 M *N*-ethylmorpholine·HCl, pH 8.0, at 20 °C.

of the reaction is rapid equilibrium random, with two dead-end complexes, as when Mg^{2+} is the activating ion [2, 3]. The agreement between the values obtained by both the proton relaxation rate measurements and the kinetic experiments is very satisfactory (Table I). The dissociation constant for $MnADP^-$ is very similar to that for $MgADP^-$, viz. 24 ($\pm$4) μM [2], which would be consistent with the metal ion not being bound directly to the enzyme in forming the ternary enzyme–metal–substrate complex. The kinetic experiments also yielded a value of 0.14 mM for the dissociation constant of the phosphoarginine–enzyme complex.

ACKNOWLEDGEMENTS

This work was supported by the National Health and Medical Research Council (W.J.O'S.) and by the Australian Research Grants Commission (B.E.C.). We thank Dr Mildred Cohn for informing us of results from her laboratory prior to publication.

REFERENCES

1 Morrison, J. F. (1973) in The Enzymes (Boyer, P. D., ed.), Vol. 8, pp. 457–486, Academic Press, New York
2 Smith, E. and Morrison, J. F. (1969) J. Biol. Chem. 244, 4224–4234
3 Smith, E. and Morrison, J. F. (1972) J. Biol. Chem. 246, 7764–7772
4 Uhr, M. L., Marcus, F. and Morrison, J. F. (1966) J. Biol. Chem. 241, 5428–5435
5 Mildvan, A. S. and Cohn, M. (1970) Adv. Enzymol. 33, 1–70
6 O'Sullivan, W. J., Marsden, K. H. and Leigh, Jr, J. S. (1973) in Physical Principles and Techniques of Protein Chemistry, Part C (Leach, S. J., ed.), pp. 245–300, Academic Press, New York
7 Buttlaire, D. H. and Cohn, M. (1974) J. Biol. Chem., in the press
8 Buttlaire, D. H. and Cohn, M. (1974) J. Biol. Chem., in the press
9 O'Sullivan, W. J., Reed, G. H., Marsden, K. H., Gough, G. R. and Lee, C. S. (1972) J. Biol. Chem. 247, 7839–7843
10 Mildvan, A. S. and Cohn, M. (1963) Biochemistry 2, 910–919
11 Reed, G., Cohn, M. and O'Sullivan, W. J. (1970) J. Biol. Chem. 245, 6547–6552

12 O'Sullivan, W. J., Virden, R. and Blethen, S. (1969) Eur. J. Biochem. 8, 562–570
13 O'Sullivan, W. J. and Cohn, M. (1966) J. Biol. Chem. 241, 3104–3115
14 Ellman, G. L. (1959) Arch. Biochem. Biophys. 82, 70–77
15 O'Sullivan, W. J. and Cohn, M. (1968) J. Biol. Chem. 243, 2737–2744
16 Reed, G. H., Diefenbach, H. and Cohn, M. (1972) J. Biol. Chem. 247, 3066–3072
17 McLaughlin, A. C., Cohn, M. and Kenyon, G. L. (1972) J. Biol. Chem. 247, 4382–4388
18 Cleland, W. W. (1963) Nature 198, 463–465
19 Flashner, M., Tamir, J., Mildvan, A. S., Meloche, H. P. and Coon, M. J. (1973) J. Biol. Chem. 248, 3419–3425