Effects of Arginine and some Analogues on the Partial Adenosine Triphosphate–Adenosine Diphosphate Exchange Reaction Catalysed by Arginine Kinase

EVOLUTIONARY DIVERGENCE IN THE MECHANISM OF ACTION OF A MONOMER AND A DIMER ARGININE KINASE

By EMMANUEL O. ANOSIKE* and DAVID C. WATTS

Department of Biochemistry and Chemistry, Guy's Hospital Medical School, London SE1 9RT, U.K.

(Received 15 December 1975)

1. Both the monomer arginine kinase from lobster muscle and the dimer arginine kinase from *Holothuria forskali* catalyse the ATP–ADP partial exchange reaction at rates equal to 3 and 0.6% of the normal rate of transphosphorylation respectively. The Mg^{2+} –nucleotide complex is the substrate for this as it is for the kinase reaction. 2. Analogues of arginine inhibit the exchange reaction of the lobster enzyme but enhance that of the *Holothuria* enzyme. 3. With the lobster enzyme NO_3^- has no effect on the exchange reaction alone and inhibit only slightly the apparent enhancement of the exchange reaction produced by the addition of arginine. This is compatible with previous findings for this enzyme that formation of the anion-stabilized dead-end complex, enzyme–arginine– $MgADP-NO_3^-$, does not occur to any marked degree. 4. About 80% of the ADP–ATP exchange reaction of the lobster enzyme remains after inhibition with iodoacetamide. This is further decreased to 65% by the addition of L-arginine, indicating that this substrate does bind to the thiol-modified enzyme. 5. It is concluded that the partial exchange reaction is a genuine phenomenon not mediated by trace amounts of arginine. From the effects of arginine and related compounds it would appear that during the normal kinase reaction the partial ATP–ADP exchange reaction is suppressed in the lobster enzyme but enhanced in the *Holothuria* enzyme. This reflects a remarkable evolutionary divergence of two homologous enzymes.

The normal mechanism of action of the phosphagen kinases is considered to be by direct phosphoryl transfer from one substrate to the other. However, there is evidence that for some of them at least part of the reaction flux proceeds via a phosphorylated enzyme intermediate. Among the arginine kinases, direct transfer appears to be the only mechanism involved for the enzyme from the king crab, *Limulus polyphemus* (Blethen, 1972), from the polychaete worm, *Sabella pavonina* (Robin *et al.*, 1971) and from the honey bee, *Apis mellifera* (Cheung, 1973). For three other monomer arginine kinases, part of the reaction flux appears to involve a phosphorylated enzyme intermediate. These are the enzyme from the European lobster, *Homarus vulgaris* (Roustan *et al.*, 1971), the Australian lobster, *Jasus verreauxi* (Uhr *et al.*, 1966) and the Australian crayfish, *Panulirus longipes* (Smith & Morrison, 1969).

There is now considerable evidence for both creatine kinase and arginine kinase that binding of the guanidine substrate to the enzyme– Mg^{2+} –nucleotide complex causes a conformational change in the enzyme that is an important part of the catalytic process

(Watts, 1973; Roustan *et al.*, 1968). The present work was carried out to see if analogues of arginine affected the ATP–ADP exchange rate with the arginine kinase from the European lobster. A few observations on the enzyme from *Holothuria forskali* are also reported because no dimeric arginine kinase has yet been studied and because of the evolutionary interest in comparing these two forms of arginine kinase.

Materials and Methods

Materials

ADP and ATP (disodium salts) were from Boehringer Corp. (London) Ltd., London W.5, U.K. Arginine solutions were prepared from arginine (free base) [Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.] and the pH was adjusted with dilute acetic acid.

[U- ^{14}C]ADP (specific radioactivity 500–600 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Iodoacetamide (BDH Chemicals Ltd., Poole, Dorset, U.K.) was purified by recrystallization from 50% (v/v) ethanol as described by Watts *et al.* (1961). LiCl (General-Purpose grade) was from BDH Chemicals Ltd.

* Present address: Department of Biochemistry, University of Nsukka, Nsukka, Nigeria.

Other chemicals were as described by Anosike *et al.* (1974).

Enzymes

Arginine kinase from lobster (*Homarus vulgaris*) muscle was prepared as described by Virden *et al.* (1965). The mol.wt. was taken as 37000 (Virden & Watts, 1966). Sea cucumber (*H. forskali*) arginine kinase (assumed mol.wt. 78500) was prepared as described by Anosike *et al.* (1974).

Enzyme assay

Arginine kinase activity was determined by measuring the acid-labile arginine phosphate formed in 10 min at 30°C in a total reaction volume of 0.4 ml as described by Virden & Watts (1966).

Partial isotope exchange

The methods of Morrison (1968) and Morrison & Cleland (1966) were used with some modifications.

For the ATP-ADP exchange, 0.5 ml of the reaction mixture contained 50 mM-Tris/acetate buffer, pH 8.6, in which was dissolved either arginine or other modifier to be tested, 2 mM-ATP, 4 mM-magnesium acetate and 40–100 µg of suitably diluted enzyme. After equilibration for 5 min at 30°C, a mixture of 1 mM-ADP (unlabelled marker) and [¹⁴C]ADP (0.025 µCi) in 0.1 ml was added to start the reaction.

To follow the exchange reaction with time, 50 µl samples were withdrawn at intervals into 50 µl portions of 100 mM-EDTA in slots on a welled plastic slab and rapidly mixed with a glass rod to stop the reaction. The control contained all components of the reaction mixture except magnesium acetate.

Chromatographic separation of ATP-ADP

Portions (10 µl) of the 'stopped' reaction mixtures were spotted on to polyethyleneimine-cellulose F plates (20 cm × 20 cm, thickness 0.1 cm), supplied by Anderman and Co. Ltd., 87–95 Tooley Street, London S.E.1, U.K., with a syringe and dried with a hair dryer. Development was by ascending liquid chromatography in the solvent system 0.4 M-formic acid/0.5 M-LiCl for 2 h. After drying in air, the ATP and ADP spots were detected by their fluorescence in u.v. light (254 nm).

The ATP spots were cut out and immersed in 15 ml of scintillator [3 g of 2,5-diphenyloxazole (PPO) and 100 mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene (POPOP) in 1 litre of AnalaR toluene]. The polyethyleneimine-cellulose was scraped from the metal foil into the solution by means of a glass rod with a flattened end and the tube contents were counted for radioactivity in a Beckman liquid-scintillation spectrometer (model 1650).

To test that 100 mM-EDTA was sufficient to chelate the Mg²⁺ present and so stop the reaction, the mixture of the sample and EDTA was spotted on a plate after further time-intervals. The plate was developed and the radioactive ATP counted as before. There was no significant increase in the ATP radioactivity after the addition of EDTA. The reproducibility of individual measurements was generally found to be approximately ±5% of the value. The percentage inhibition values quoted were determined from the initial slopes of the exchange reactions.

Thiol-modified lobster arginine kinase

The 'essential' thiol group of this enzyme was made to react at pH 7.0 with iodoacetamide as described by Roustan *et al.* (1971). This modified enzyme when tested showed less than 0.2% activity, compared with the native sample.

Rates of inactivation of the enzymes by iodoacetamide

To find the correct range of concentrations of the inhibitor to use, 0.05 ml of appropriately diluted enzyme was incubated with final concentrations of the inhibitor varying from 0.1 mM to 5.0 µM for 10 min at 30°C. At the end of this time, the reaction was stopped by the addition of 0.15 ml of 10 mM-cysteine, pH 8.5. Each tube was then assayed for residual enzyme activity.

To follow the loss of activity with time, 0.34 µg (23 nm) of lobster arginine kinase or 0.45 µg (14.3 nm) of *H. forskali* enzyme was used for each assay in 0.4 ml. The enzyme was incubated with the appropriate concentration of iodoacetamide at 30°C and pH 8.5. At suitable time-intervals, 0.1 ml portions were transferred to 0.15 ml portions of 10 mM-cysteine and 0.05 ml of 40 mM-arginine was added. The assay reaction for residual activity was started with 0.1 ml of 16 mM-MgATP and carried out as described above.

Results

ATP-ADP exchange reaction

This partial exchange reaction catalysed by arginine kinase is shown by eqns. (1) and (2):

The rate of exchange may be followed from the formation of radioactive ATP by incorporation of a phosphoryl group into radioactive ADP (ADP*). This is shown for the lobster enzyme in Fig. 1 and confirms the finding of Roustan *et al.* (1971). Fig. 2 shows that the dimeric arginine kinase of *Holothuria forskali* also catalyses this partial exchange reaction.

Fig. 1. Mg^{2+} -activated ATP-ADP exchange reaction catalysed by lobster arginine kinase and its modification by analogues of arginine

The experimental procedure is described in the Materials and Methods section. Each reaction mixture contained $46.2 \mu\text{g}$ of enzyme at pH 8.6 and 30°C . ●, Exchange reaction in the absence of arginine or its analogues; ○, +20mM-L-ornithine; ▲, +13mM-argininic acid; △, +20mM-L-isoleucine. See the text for definition of ATP*.

Fig. 2. Mg^{2+} -activated ATP-ADP exchange reaction catalysed by arginine kinase from *Holothuria forskali*

The experimental procedure is described in the Materials and Methods section. Each reaction mixture contained $100 \mu\text{g}$ of enzyme at pH 8.6 and 30°C . ●, Exchange reaction in the absence of arginine or its analogues; ○, +20mM-L-ornithine; ▲, +13mM-argininic acid; △, +20mM-L-citrulline.

For both enzymes no exchange occurred if Mg^{2+} ions were omitted from the reaction mixture.

Effect of arginine analogues

Because of the known effect of the guanidine substrate in causing a conformational change in these enzymes, the effects of some analogues of arginine were investigated. No analogue is a substrate for the kinase reaction. Fig. 1 shows that the addition of 20mM-L-ornithine inhibits the initial exchange rate of the lobster enzyme by about 50%; 20mM-L-isoleucine and 13mM-argininic acid each caused about 25% inhibition. With the *Holothuria* enzyme arginic acid had a profound enhancing effect on the exchange rate, whereas with L-ornithine a small enhancement was observed (Fig. 2). L-Citrulline, although a known inhibitor of the enzyme, did not affect the exchange rate (Fig. 2).

Effect of nitrate ions

The lobster enzyme has been suggested to form only a weak dead-end complex, enzyme-MgADP-arginine- NO_3^- (Anosike & Watts, 1975). Fig. 3 shows experiments to determine the effect of NO_3^- on the ATP-ADP exchange reaction. By itself NO_3^- is without effect. The addition of a small amount of

Fig. 3. Effects of NO_3^- and arginine on the ATP-ADP exchange reaction catalysed by lobster arginine kinase

Conditions were as in Fig. 1 except that $43.5 \mu\text{g}$ of enzyme were used for each experiment. ●, No additions; ○, +25mM- NaNO_3 ; ▲, +0.1mM-L-arginine; △, +25mM- NaNO_3 and 0.1mM-L-arginine.

Fig. 4. Plots of the inhibition of arginine kinases by iodoacetamide by using the procedure of Hollenberg *et al.* (1971)

●, Lobster arginine kinase (21 nM) inhibited by iodoacetamide used at 12.5, 25, 37.5 and 50 μ M. ○, *Holothuria* arginine kinase (14 μ M) inhibited by iodoacetamide used at 12.5, 25, 31.25 and 62.5 μ M. Reactions were carried out at pH 7.0 and 30°C as described in the Materials and Methods section. Residual activity after time t in the presence of the inhibitor, i , was determined from a progress curve for each set of reaction conditions.

arginine would be expected greatly to speed up the apparent exchange reaction, because this may now proceed via the pathway with phosphoarginine as intermediate (eqns. 3 and 4).

Fig. 3 shows that the apparent exchange reaction is greatly accelerated by the addition of arginine and that this enhancement is slightly decreased by the addition of NaNO_3 as well.

Carboxymethylated arginine kinase

Both arginine kinases are readily inactivated by iodoacetamide at pH 7.0 and 30°C. The residual activity after alkylation is no greater than 0.2% of that of the native enzyme. The number of moles of inhibitor reacting per active site was determined as described by Hollenberg *et al.* (1971). This is indicated by the slopes of the plots in Fig. 4, which is 1.02 for lobster arginine kinase and 0.90 for the *Holothuria* kinase. Thus for both enzymes only one molecule of iodoacetamide reacts per catalytic site with a resulting loss of enzyme activity.

Fig. 5. Modification of the Mg^{2+} -activated ATP-ADP exchange reaction of iodoacetamide-inhibited lobster arginine kinase by arginine and related compounds

●, Native enzyme in the absence of arginine or analogue; ○, enzyme inhibited by iodoacetamide as described in the Materials and Methods section; ▲, inhibited enzyme + 8 mM-L-arginine; Δ, inhibited enzyme + 20 mM-L-ornithine. Other details are as in Fig. 1.

ATP-ADP exchange reaction of alkylated lobster arginine kinase

The ATP-ADP exchange rate of the alkylated enzyme was about 80% of that for the native enzyme (Fig. 5). The addition of arginine, in contrast with what was found with the native enzyme, now further decreased the exchange rate to about 65% of that for the native enzyme (Fig. 5). With added ornithine, however, no modification of the exchange rate was now observed.

Discussion

The results presented here confirm the finding by Roustan *et al.* (1971) that lobster arginine kinase appears to catalyse an ATP-ADP exchange reaction in the presence of Mg^{2+} . Removal of the Mg^{2+} by chelation stops the exchange reaction, indicating that the Mg^{2+} -nucleotide complex is the true substrate for the exchange reaction as well as for phosphoryl transfer to arginine. However, Fig. 3 shows that it is important to exclude the possibility of an exchange reaction being simulated by contamination of the preparation with small amounts of the guanidine substrate according to the reactions shown in eqns. (3) and (4). For the lobster enzyme this is particularly so because analogues of arginine slow down the exchange rate (Fig. 1) and might be inferred to act by competition with traces of free arginine. In fact, the partial exchange does not appear to be arginine-dependent

because it is still found with the alkylated enzyme and under these conditions arginine now decreases the exchange rate. Further support for a genuine partial exchange comes from the effect of NO_3^- , which inhibits exchange when arginine is deliberately added to the reaction mixture, but is without effect on the exchange that occurs when arginine is not known to be present.

The effects of arginine analogues on the native lobster enzyme, and of arginine on the alkylated enzyme, are all to decrease the partial exchange rate. It is noteworthy that Roustan *et al.* (1968) report that alkylation of lobster arginine kinase with iodoacetamide produces a difference spectrum similar to that observed when arginine is added to the native enzyme. Bearing in mind that alkylation itself also decreases the partial exchange rate (Fig. 5) all these data point to the idea that whereas the Mg^{2+} -activated ATP-ADP exchange can occur in the absence of arginine, it is either inhibited or strongly suppressed under conditions of normal phosphoryl transfer. On the basis of the partial exchange in the absence of the second substrate, Smith & Morrison (1969) concluded that for arginine kinase from *Panulirus longipes* less than 1% of the total reaction flux proceeded via a Ping Pong mechanism, and Roustan *et al.* (1971) came to similar conclusions about the lobster enzyme. Our own data indicate a value of just over 3%. Now that the effect of arginine can also be anticipated, it seems that for the lobster enzyme at least, the proportion of the reaction flux of the transphosphorylation reactions proceeding via a phosphorylated enzyme intermediate would be negligible.

Roustan *et al.* (1968, 1971), on the basis of difference spectral measurements and the inability of alkylated lobster arginine kinase to carry out an arginine-phosphoarginine exchange reaction, concluded that the alkylated enzyme did not bind arginine. In contrast, the present results (Fig. 5) show that arginine will modify the rate of ATP-ADP exchange mediated by alkylated lobster arginine kinase. This problem of the binding of the guanidine substrate in relation to chemical modification of the reactive thiol group requires further attention.

The *Holothuria* arginine kinase consists of two subunits that will hybridize with mammalian creatine kinase (Watts *et al.*, 1972). Fig. 4 shows that in common with other phosphagen kinases it contains one reactive thiol group per subunit, the alkylation of which by iodoacetamide causes almost complete loss of catalytic activity. Preliminary observations on the Mg^{2+} -activated ATP-ADP partial exchange reaction (Fig. 2) indicate that it represents only about 0.6%

of the total reaction flux for an enzyme with a specific activity of $174 \mu\text{mol/min per mg}$. However, unlike the lobster enzyme the presence of the non-substrate arginine analogue, argininic acid, increases the exchange rate by more than 350%. Slight enhancement was also obtained by ornithine, suggesting that a positive charge on the δ -nitrogen atom may be important, since although citrulline was without effect it is known to inhibit catalysis (E. O. Anosike, unpublished work). By analogy with the lobster enzyme it might be inferred that the presence of arginine would enhance that part of the transphosphorylation reaction proceeding via a phosphorylated enzyme intermediate. This represents a remarkable divergence in the catalytic behaviour of two enzymes that may reasonably be assumed to be homologous. Whether or not this difference relates to the formation of an enzyme dimer (rabbit muscle creatine kinase shows no partial exchange reaction) may emerge with further study.

E. O. A. is very grateful to the Commonwealth Scholarship Commission of the United Kingdom for a scholarship grant.

References

- Anosike, E. O. & Watts, D. C. (1975) *Biochem. J.* **149**, 387–395
- Anosike, E. O., Moreland, B. H. & Watts, D. C. (1974) *Biochem. J.* **145**, 535–543
- Blethen, S. L. (1972) *Arch. Biochem. Biophys.* **149**, 244–251
- Cheung, A. C. (1973) *Arch. Biochem. Biophys.* **154**, 28–39
- Hollenberg, P. F., Flashner, M. & Coon, M. J. (1971) *J. Biol. Chem.* **246**, 945–953
- Morrison, J. F. (1968) *Anal. Biochem.* **24**, 106–111
- Morrison, J. F. & Cleland, W. W. (1966) *J. Biol. Chem.* **241**, 673–683
- Robin, Y., Klotz, C. & Thoai, N. V. (1971) *Eur. J. Biochem.* **21**, 170–178
- Roustan, C., Kassab, R., Pradel, L. A. & Thoai, N. V. (1968) *Biochim. Biophys. Acta* **167**, 326–338
- Roustan, C., Pradel, L. A., Kassab, R. & Thoai, N. V. (1971) *Biochim. Biophys. Acta* **250**, 103–116
- Smith, E. & Morrison, J. F. (1969) *J. Biol. Chem.* **244**, 4224–4234
- Uhr, M. L., Marcus, F. & Morrison, J. F. (1966) *J. Biol. Chem.* **241**, 5428–5435
- Viriden, R. & Watts, D. C. (1966) *Biochem. J.* **99**, 162–172
- Viriden, R., Watts, D. C. & Baldwin, E. (1965) *Biochem. J.* **94**, 536–544
- Watts, D. C. (1973) *Enzymes*, 3rd edn., **8**, 383–455
- Watts, D. C., Rabin, B. R. & Crook, E. M. (1961) *Biochim. Biophys. Acta* **48**, 380–388
- Watts, D. C., Focant, B., Moreland, B. H. & Watts, R. L. (1972) *Nature (London) New Biol.* **237**, 51–53