The Use of Arginine Analogues for Investigating the Functional Organization of the Arginine-Binding Site in Lobster Muscle Arginine Kinase

ROLE OF THE 'ESSENTIAL' THIOL GROUP

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1. The nature of arginine binding to lobster arginine kinase and the extent of its possible involvement with the 'essential' thiol group of the enzyme has been investigated with some inhibitory analogues of arginine. 2. Most of the analogues inhibit competitively, although mixed inhibition may occur if the α -carboxy group or α -amino group is absent. 3. The K_i values indicate that strength of binding depends on the length of the carbon chain (L-isoleucine > L-valine > L- α -aminobutyrate > L-alanine) and the integrity of the substituents on the α -carbon atom (L-arginine > agmatine and L-ornithine > putrescine). The guanidino group probably contributes little to substrate binding, but a positive charge near the δ -nitrogen atom appears to be important (L-ornithine > L-citrulline > L- α -aminobutyrate). A cyclic analogue, 2-carboxymethyl-3-oxo-2,3,5,6,7,8-hexahydro-1*H*-imidazo[1,2-*a*][1,3]diazepine-8-carboxylic acid, has a low K_i value similar to that of an equivalent straight-chain form, suggesting that arginine probably binds in a folded configuration. 4. The aliphatic L-amino acids give enzyme difference spectra similar to that with L-arginine and the integrity of the α -carboxy and α -amino groups appears to be a minimal but not sufficient requirement for this, as L-ornithine gives an atypical difference spectrum. A difference spectrum is interpreted as indicating an enzyme conformational change. No difference spectrum was observed with methylguanidine. 5. The ability of aliphatic α -L-amino acids to protect against inhibition by 5,5'-dithiobis-(2-nitrobenzoic acid) is proportional to the number of atoms in the carbon chain and inversely proportional to K_i . Ornithine gives greater protection than citrulline; analogues lacking the α -amino groups also protect. Agmatine, lacking the α -carboxy group, did not protect. 6. It is concluded that it is unlikely that the 'essential' thiol group in the enzyme interacts with any part of the arginine molecule during catalysis except, possibly, the α -carboxyl group.

Interest in the amino acid side chains that are important for the catalytic reaction of the phosphagen kinases has centred on the role of the pH-independent reactive thiol group that appears to be a common feature of these enzymes. This also appears to be of fundamental functional importance in that it resides in a peptide, the sequence of which is conserved, with only minor variations, regardless of species

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and the nature of the guanidine substrate (reviewed by Watts, 1975). Several mechanisms have been proposed for arginine kinase involving the essential thiol group (Virden & Watts, 1966; Mildvan & Cohn, 1970). Work by Thoai and his coworkers (Roustan *et al.*, 1968, 1970) led to the conclusion that, in lobster arginine kinase, this thiol group formed a contact with the substrate, arginine, as part of the catalytic process. More specifically, it was suggested that interaction occurred with the α -

amino group and that the integrity of the substituents on the α -carbon atom of arginine was important for activity (Roustan *et al.*, 1970).

A theoretical difficulty against accepting this conclusion for the role of the thiol group is that it is not readily compatible with the idea of a common evolutionary origin for the phosphagen kinases (Watts, 1965, 1971, 1975; Thoai, 1968). The anionic substituent on the α -carbon atom of the guanidine substrate may be a simple carboxy group, as in creatine, a sulphinic or sulphonic acid group, as in hypotaurocyamine and taurocyamine, or a disubstituted phosphate group, as in opheline and lombricine. Further, only arginine has an α -amino group. It seemed difficult to equate such structural diversity in this region of the substrate molecule with a lack of corresponding variability in its suggested binding site, and the hypothesis provided no obvious explanation of why a thiol group with such unusual properties should be so strictly conserved in an almost constant peptide sequence. This stimulated the present reinvestigation of the nature of the arginine kinase-arginine interaction.

Materials and Methods

Materials

ATP (disodium salt) was obtained from Boehringer Co. (London), London W.5, U.K. The sodium salts of ADP, IDP and AMP were obtained from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K., as were all L- α -amino acids, methylguanidine, creatine, putrescine (1,4-diaminobutane), agmatine sulphate (4-amino-1-guanidinobutane sulphate), argininic acid and 5,5'-dithiobis-(2-nitrobenzoic acid). Agmatine sulphate solution was converted into the acetate by treatment with a stoichiometric amount of a solution of barium acetate and filtering off the insoluble BaSO₄. Glycocyamine was from Koch-Light Laboratories, Colnbrook, Bucks., U.K., and recrystallized several times from hot water. Taurocyamine was prepared as described by Watts & Moreland (1970). Glycocyamylglycine was prepared as described by Virden & Watts (1964). Argininosuccinic acid and its anhydrides were obtained as described by Lee & Pollitt (1972).

Argininic acid was found by chromatography to contain traces of arginine as contaminant. Before use, it was purified by chromatography on Amberlite CG 120 (BDH, Poole, Dorset, U.K.). Approx. 10g of the resin was thoroughly washed with double-distilled water, and its pH (approx. 5.0) was adjusted to 7.0 with dilute NaOH. The suspension of resin was poured into a column (1.2 cm $\times$ 6 cm) and 10 ml of the solution of argininic acid in water (100 mM) was applied, eluted with water and was collected in 2 ml fractions. Argininic acid was

detected in the fractions by spotting a sample (2–5 μ l) of each fraction on to Whatman no. 1 filter paper and by staining for guanidines with 1% α -naphthol in 8% (w/v) NaOH, followed by 0.1% diacetyl in water, followed by heating in an oven at 100°C for 3 min (Smith, 1958). Contents of the tubes that gave positive results were pooled. The absence of arginine from the product was confirmed by t.l.c. (Eastman Chromatogram sheet; 100 μ m thick silica gel), with butanol/acetic acid/water (1:2:2, by vol.) as the developing solvent.

Other chemicals and purified lobster arginine kinase were as described by Anosike *et al.* (1975).

Methods

Enzyme assay. For the inhibition studies, enzyme activity was measured by determining the acid-labile arginine phosphate formed in 10 min at 30°C as described by Virden & Watts (1964), except that magnesium acetate was used instead of MgSO₄. The final reaction mixture (total volume 0.4 ml) contained: 0.1 ml of a solution containing 16 mM-magnesium acetate and 16 mM-disodium ATP, adjusted to pH 8.6 with dilute NaOH before making up to the final volume; 0.15 ml of 100 mM-Tris/acetate buffer, pH 8.6; 0.1 ml of L-arginine at the desired concentration; 0.05 ml of suitably diluted enzyme. In appropriate experiments the buffer solution also contained the arginine analogue under investigation and, if necessary, the pH was readjusted to 8.6 before the solution was made up to the final volume.

The enzyme was equilibrated at 30°C with the Tris/acetate buffer plus modifier for 5 min before the addition of L-arginine. MgATP²⁻ was added after a further 5 min equilibration to start the reaction. The rest of the assay procedure was as described by Virden & Watts (1964). The rate of reaction was essentially linear with time over the incubation times used. The effect of the analogue on the total ionic strength of the assay mixture was not sufficiently significant to affect the enzyme reaction rate from this cause.

Protection by analogues against inhibition by 5,5'-dithiobis-(2-nitrobenzoic acid). The general procedure described by Roustan *et al.* (1968) was followed. The enzyme solution (0.025 ml containing 0.3–0.4 μ g of protein) was preincubated for 5 min with 0.15 ml of a solution containing the analogue (43.75 mM) dissolved in 50 mM-Bicine [NN-bis-(2-hydroxyethyl)glycine] buffer, pH 8.5. Inhibition was started by adding 0.025 ml of 5,5'-dithiobis-(2-nitrobenzoic acid) dissolved in 50 mM-Bicine buffer, pH 7.2, to give a final inhibitor concentration of 0.25 μ M.

After incubation at 30°C for 20 min, 0.1 ml of 20 mM-arginine solution was added, and the residual enzyme activity was estimated by the addition of 0.1 ml of MgATP²⁻ solution (prepared as described

above and a further incubation for 15 min at 30°C. The assay reaction was then stopped and the phosphoarginine was measured by the method of Virden & Watts (1964). All experiments were carried out in duplicate.

Difference spectra. Measurements were made at pH 7.8 in a Cary 16S recording spectrophotometer thermostatically maintained at 25°C and with split-path-length cells, each half-cell 4.5 mm path-length, as described by Focant & Watts (1973). All solutions were filtered through Millipore Swinnex-25 filters immediately before use. The enzyme was pre-dialysed against 10 mM-Tris/acetate buffer, pH 7.8, to remove Bicine buffer, which absorbs in the u.v. region.

Results and Discussion

Analogues of arginine were chosen to provide variation in the four parts of the molecule, i.e. the α -

amino group, the α -carboxy group, the carbon chain and the guanidino group (Table 1). The structures of the anhydrides of argininosuccinic acid (III) are shown by structures (I), II and (IV), the numbering system of Lee & Pollitt (1972) being followed. Lobster arginine kinase appears to be highly specific for L-arginine as substrate and none of the analogues gave detectable activity even when used at high concentrations. This supports the observation that the integrity of the substituents on the α -carbon atom is a prerequisite for enzyme activity (Roustan *et al.*, 1970).

Inhibition by analogues of L-arginine

Inhibition by arginine analogues was investigated by using two or three concentrations of the analogue as indicated in Table 1, each with the following concentrations of arginine: 2.0, 1.0, 0.67, 0.5, 0.4, 0.33 and 0.25 mM. The concentrations of magnesium acetate and ATP were held constant at 4 mM, as described in the Methods section. The resulting data were analysed by plots of $1/v$ against [I] and [S]/ v against [I] (Cornish-Bowden, 1974) to determine the type of inhibition and to evaluate K_i and K'_i , the dissociation constants for the EI and EIS complexes respectively. When the inhibition type was competitive by this criterion, K_i was also determined, for comparison (see Table 1), from the intercepts of double-reciprocal plots. The two methods usually gave reasonable agreement. The K_m for arginine was determined in the same way from the control curves (analogue omitted) and this value is also listed in Table 1. The analogues were investigated either singly or two at a time, and a control curve was run with each experiment.

All the analogues that possessed both an α -amino and an α -carboxy group inhibited competitively with respect to arginine. This situation was unaffected by variation in the chain length of the carbon backbone or by the absence of the guanidino group. Alanine, like the other aliphatic amino acids, was assumed to inhibit competitively, as it was too weak an inhibitor for this to be determined with any degree of confidence. Glycocyamylglycine, glycocyamine and taurocyamine gave weak competitive inhibition, although lacking the α -amino group. Agmatine and putrescine also lack the α -amino group, but gave mixed inhibition (Cornish-Bowden, 1974), as did methylguanidine, which has neither α -amino nor α -carboxy groups. As might be expected, the analogues most like arginine are good competitive inhibitors, and the ability to act as an inhibitor becomes progressively poorer as the analogue becomes less like the substrate. Thus the specificity of substrate binding appears to show a high degree of dependence on the exclusion geometry of the binding site. However, the finding that agmatine binds well but is not a substrate suggests that substrate binding is also asso-

Table 1. *Effects of L-arginine and some analogues of L-arginine on lobster muscle arginine kinase*

	Groups present in the molecule			Carbon chain length	Type of inhibition and number of concentrations of inhibitor used	Plot used and kinetic constant determined	Kinetic constant (mm)				Protection against inhibition by 5,5'-dithiobis-(2-nitrobenzoic acid) (%)
	α -Carboxy	α -Amino	Guanidino				[S]/V against K_i' for K_i	1/V against [I] for K_i	1/V against 1/[S] for K_m (arginine)	1/[S] for K_m (arginine)	
L-Ornithine	+	+	$-\text{NH}_2$	4	Competitive (2)		—	23	23	1.3	87.5
L-Citrulline	+	+	$-\text{NHC}(\text{ONH}_2)$	4	Competitive (3)		—	70	74	1.3	68.5
L-Isoleucine	+	+	H	4 (+1)	Competitive (3)		—	38	54	1.4	100.0
L-Valine	+	+	H	3 (+1)	Competitive (2)		—	70	81	1.4	82.0
L- α -Aminobutyrate	+	+	H	4	Competitive (2)		—	200	180	1.4	76.8
L-Alanine	+	+	H	2	? Competitive (2)		—	—	>200	1.3	N.T.
Agmatine (4-amino-1-guadinobutane)	H	+	+	4	Mixed (3)		90	29	—	1.1	0
Putrescine (1,4-diaminobutane)	H	+	$-\text{NH}_2$	4	Mixed (2)		80	65	—	1.1	N.T.
Glycocyamylglycine	+	H	+	4*	Competitive (2)		—	65	67	1.4	N.T.
Creatine	+	H	+	1 (+1)	Mixed (3)		68	45	—	1.8	67.5
Glycocyamine	+	H	+	1	Competitive (2)		—	110	128	1.3	N.T.
Taurocyamine	$-\text{SO}_3\text{H}$	H	+	2	Competitive (2)		—	175	168	1.4	N.T.
Methylguanidine	H	+	+	1	Mixed (3)		160	120	—	1.4	N.T.
L-Arginine	+	+	+	4	—		—	—	—	—	89.0
L-Homoarginine	+	+	+	5	—		—	—	—	—	83.5
L-Arginonic Acid	+	+	+	4	—		—	—	—	—	68.5
L-Argininosuccinic anhydride (I)	+	+	+	4	Competitive (2)		—	26	20	1.3	N.T.
L-Argininosuccinic anhydride (II)	+	+	+	4	Mixed (3)		>100	>40	—	1.3	N.T.
L-Argininosuccinate (III)	+	+	+	4	Mixed (2)		35	26	—	1.2	N.T.
L-Argininosuccinic anhydride (IV)	+	$-\text{N}=\text{N}$	+	4	Competitive (2)		—	32	26	1.5	N.T.

* Including a peptide bond.

The essential features of the analogues are indicated by a plus sign if the group is present or by the atom or group that replaces it. The number of atoms in the carbon chain is also indicated with additional side-chain methyl groups given in parentheses. Enzyme activity was determined at pH 8.6 and 30°C with fixed concentrations of ATP and magnesium acetate and seven concentrations of arginine, as given in the text, in the absence of analogue and in the presence of the number of concentrations of analogue given in the Table. These were: (2) 25 and 37.5 mM; (3) 12.5, 25 and 37.5 mM except for compounds 11–(IV), where owing to limited availability of material the concentrations were (2) 6.25 and 9.375 mM; (3) 3.125, 6.25 and 9.35 mM. The type of inhibition of enzyme activity was determined graphically (Cornish-Bowden, 1974). The plots were also used to derive inhibition constants where K_i is the dissociation constant for the EI complex and K_i' that for the EIS complex. K_i and K_m values for arginine determined from double-reciprocal plots are also listed for comparison. The percentage protection against inhibition by 5,5'-dithiobis-(2-nitrobenzoic acid) was calculated as described in the text from the residual enzyme activity found after incubation with arginine analogues plus inhibitor compared with that found after incubation with the analogue alone. A percentage protection of 100 indicates no loss of enzyme activity in the presence of the inhibitor. Abbreviation used: N.T., not tested.

ciated with an enzyme conformational change and this is supported by the difference spectra and thiol-protection experiments described below. Substituents around the α -carbon atom of the substrate appear to be important for this process. The analogues giving mixed inhibition may do so as the result of a non-productive conformation change that cannot be reversed by arginine.

For lobster arginine kinase it is well known that the steric arrangement of the substituents around the α -carbon atom is a crucial requirement for an active substrate. D-Arginine is not a substrate for the lobster enzyme (Virden & Watts, 1964). On the other hand it is of mechanistic interest that arginine kinases of flatworms (*Turbellaria*) and some polychaete annelids will phosphorylate D-arginine, the latter species at a rate similar to that found with the normal L-isomer (Virden & Watts, 1964; Robin *et al.*, 1969, 1971, 1975). Among these groups, species with enzymes that phosphorylate arginine analogues modified at the α -amino group are also found (Virden & Watts, 1964). Hence it seems that the involvement of substituents around the α -carbon atom is an adaptive feature rather than a fundamental part of the transphosphorylation process.

The binding of arginine to arginine kinase results in a difference spectrum (Roustan *et al.*, 1968). This could indicate the occurrence of a conformational change that also results in protection of the reactive thiol group as described below. The neutral amino acids, such as L-isoleucine, L-valine and L- α -aminobutyrate, give difference spectra which, like that for arginine, show two minima, one at 287nm and one at 279nm (Fig. 1). Thus a common mode of binding may be inferred in accord with the observed competitive inhibition. Agmatine, unlike arginine, gives a single broad trough with a minimum at 278nm (Fig. 1; Roustan *et al.*, 1968) and shows mixed inhibition. L-Ornithine also gives a single broad trough (Fig. 1c), but nevertheless inhibits competitively. Methylguanidine gives no difference spectrum at all, supporting the view that induction of the conformational change is limited to the substituents on the α -carbon atom.

It may be concluded that the precise requirements for initiating the correct conformational change in the lobster enzyme are complex and show a dependence on all the features of the molecular architecture of arginine for the formation of a functional substrate-binding site on the enzyme. This explains the absolute specificity of this enzyme for L-arginine as the natural substrate.

It has been found, by using analogues of creatine with creatine kinase, that the steric orientation of the guanidino group is important for substrate binding and for phosphoryl transfer to occur (Rowley *et al.*, 1971). Table 1 shows that L-citrulline is a weaker inhibitor of arginine kinase than compounds (I)–

Fig. 1. Difference spectra for the binding of analogues of L-arginine to arginine kinase

Measurements were carried out in 0.1 M-Tris/acetate buffer, pH 8.6. Before mixing, one compartment of each split-path-length cell contained 1 ml of 10 mM-L-arginine or analogue, and the other contained 1.6 mg of enzyme in 1 ml. The difference spectra obtained after mixing the test cell were: (a) L-arginine; (b) L-isoleucine; (c) L-ornithine; (d) agmatine; (e) L- α -aminobutyrate. Each division of the ordinate represents 0.001 A unit.

(IV), in which the integrity of the guanidino group, although heavily substituted, has been retained. Compounds (II) and (III) carry substituents that project into the region occupied by the transferable phosphoryl group and inhibit non-competitively; compounds (I) and (IV) carry substituents that do not so project and retain the competitive character associated with the presence of an α -amino group, an α -carboxy group and a guanidino group in the molecule (Table 1).

The low K_i value of compound (I) suggests that the steric restrictions imposed by the lactam ring are compatible with effective binding. Introduction of a similar five-membered ring structure into creatine (V) raised the K_m from 5 to 25 mM and only slightly decreased effectiveness as a substrate for creatine kinase under V_{max} conditions (Rowley *et al.*, 1971).

A positive charge on N^b may be important for substrate binding. This is suggested by the much

lower K_i for L-ornithine than for L-citrulline or L- α -aminobutyrate (Table 1). Again, conformational changes probably accompany binding, since L-ornithine is a better inhibitor than L-isoleucine of the partial ATP-ADP exchange reaction of native lobster arginine kinase, but both are without effect on the exchange catalysed by the enzyme blocked by iodoacetamide (Anosike & Watts, 1976).

The carbon chain probably makes the most important contribution to substrate binding. Table 1 indicates the existence on the enzyme of a large hydrophobic area that will readily accommodate branched-chain amino acids as part of the arginine-binding site. Thus the K_i values of a series of inhibitors of different carbon chain length but with no terminal positive charge runs: L-isoleucine < L-valine < L- α -aminobutyrate < L-alanine. Similarly, the simple addition of a methyl group to glycocyamine [*N*-(aminoiminomethyl)glycine] to give creatine decreases the K_i to less than half. Retaining the overall chain length of arginine but with the introduction of a peptide bond to give hydrophilic character markedly increases the K_i value, as shown for glycocyamylglycine (Table 1).

The finding that the K_i value of compound (IV), 2-carboxymethyl-3-oxo-2,3,5,6,7,8-hexahydro-1*H*-imidazo[1,2-*a*][1,3]diazepine-8-carboxylic acid, is not very different from that of compound (I) suggests that the cyclical structure is compatible with effective binding. Compound (IV) is also a competitive inhibitor, suggesting that it has a configuration very similar to that of enzyme-bound arginine. N^c may here fulfil the role of the α -amino group of arginine. This idea that arginine binds to the enzyme in a folded configuration also emerges from the work of Moreland (1974), in which the effect of another arginine analogue on molluscan arginine kinases was investigated. It explains the marked decrease in affinity produced by the introduction of an inflexible region in the molecule, as in glycocyamylglycine, as well as by the increase in hydrophilic character. It also explains the unexpectedly high affinity for creatine, which is greater than expected from its short chain length.

Protection against inhibition by 5,5'-dithiobis-(2-nitrobenzoic acid)

The ability of arginine and various analogues to protect arginine kinase against inhibition by 5,5'-dithiobis-(2-nitrobenzoic acid) is summarized in Table 1. The protecting agents were each used at a concentration of 37.5 mM rather than 10 mM as used by Roustan *et al.* (1968). When computing the percentage protection, correction had to be made for the inhibition caused by the presence of the analogue in the subsequent assay for residual enzyme activity. For this purpose measurements were made of A , enzyme activity alone; B , in the presence of

inhibitor; C , in the presence of protecting agent; and D , in the presence of inhibitor and protecting agent.

Then:

$$\text{the percentage protection} = \frac{d-b}{c} \times 100$$

where $b = (B/A) \times 100$, $c = 100 - b$, and $d = (D/C) \times 100$, and A , B , C and D are the experimental values found in the reaction mixtures indicated above.

Both arginine and homoarginine effectively protected against inhibition. In accord with the finding of Roustan *et al.* (1968), strong protection was obtained by aliphatic amino acids and the order of effectiveness for L-isoleucine, L-ornithine, L-valine and L-citrulline is in reasonable agreement with the efficiency of binding, as inferred from the K_i values. With L- α -aminobutyrate, an anomalously large amount of protection relative to that expected from the K_i value was obtained. L-Ornithine and L-isoleucine were at least as effective as L-arginine (Table 1). From these data and bearing in mind that the analogues protecting the thiol group may show competitive or mixed inhibition, may or may not give difference spectra like that of L-arginine with arginine kinase and need not possess the guanidino group, it must be inferred that any interaction between the reactive thiol group on the enzyme and the guanidino group on the substrate is unlikely. If all phosphagen kinases do indeed have a common evolutionary origin (Watts, 1971), it must also be concluded that proposed mechanisms of action for the phosphagen kinases in which this interaction occurs are highly improbable (Watts & Rabin, 1962; Virden & Watts, 1966; Mildvan & Cohn, 1970).

L-Argininic acid, which has the α -amino group replaced by a hydroxy group, gave good protection, contrary to the report by Roustan *et al.* (1968), as did creatine, which lacks an α -amino group. On the other hand agmatine, which lacks the α -carboxy group, gave no measurable protection, although it has a low K_i value (Table 1). This is in accord with the finding by Roustan *et al.* (1968) that agmatine did not protect against inhibition by iodoacetamide. These findings render improbable a proposed mechanism in which the α -amino group of L-arginine hydrogen-bonds to the reactive thiol group and thereby promotes a nucleophilic attack by a nearly histidine residue on the transferable phosphoryl group of ATP (Roustan *et al.*, 1970).

The most probable role of the reactive thiol group would seem to be that of mediating a conformational change in the catalytic process. As noted by Moreland (1974), it is only the anionic group attached to the α -carbon atom (or its equivalent) that is a constant feature of all phosphagens, although, as mentioned in the introduction, this may

vary from a carboxy group to a disubstituted phosphate. The possibility that the conformational change may occur as a result of repulsion between the thiolate anion and the anionic substituent on the α -carbon atom of the phosphagen has still to be excluded.

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