Effects of Anions on a Monomeric and a Dimeric Arginine Kinase

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1. Some effects of anions on the rates of phosphoarginine synthesis by monomeric (lobster) and by dimeric (*Holothuria forskali*) arginine kinases are reported. 2. As with creatine kinase, acetate ions activate both enzymes: Cl^- was also found to activate both although this was an inhibitor of creatine kinase. 3. NO_3^- inhibits the lobster enzyme. Inhibition is of the mixed type with respect to MgATP . $K_i > K_i'$ and $K_s > K_s'$ indicating that the presence of NO_3^- promotes the binding of substrate and vice versa. 4. NO_3^- alone has no effect on the difference spectrum of the lobster enzyme but in the presence of arginine, MgATP , MgADP , MgAMP or MgIDP the difference spectrum is greatly enhanced. A profound effect on the ionization state of tyrosine residues is inferred. 5. With the *Holothuria* enzyme low concentrations of NO_3^- activate in a manner that is competitive with arginine. Higher concentrations cause inhibition of the mixed type with respect to arginine in a similar manner to that found with MgATP for the lobster kinase. 6. Of a range of anions tested only NO_3^- and NO_2^- enhanced the inhibition of enzyme activity by MgADP , indicating the formation of a pseudo-transition-state dead-end complex, enzyme-arginine- NO_3^- - MgADP . The effect was essentially independent of temperature with the *Holothuria* enzyme, but with the lobster enzyme was much less marked and temperature dependent. The difference may reflect the different stabilities of the monomer and dimer enzymes, although with neither arginine kinase is the stabilization of the dead-end complex as marked as is found with creatinine kinase.

Milner-White & Watts (1971) found that a strong inhibition of rabbit muscle creatine kinase was caused by NO_3^- ions in the presence of the dead-end complex, creatine-enzyme- MgADP . It was suggested that the planar anion acted by mimicking the transferable phosphoryl group in a transition stage in the reaction and thereby locked the two substrates together. The effectiveness of other anions as inhibitors was related to the extent to which they could behave like NO_3^- ions. Detailed proton-relaxation-rate studies by Cohn and co-workers confirmed and extended this hypothesis (McLaughlin & Cohn, 1972; Reed *et al.*, 1972), and recently further support has come from investigations with the fluorescent probe 8-anilino-naphthalene-1-sulphonate (McLaughlin, 1974). Creatine kinases from other species (Simonarson & Watts, 1972; Focant & Watts, 1973; W. R. Chegwiddden & D. C. Watts, unpublished work, reviewed by Watts, 1973) were all found to be inhibited in the same way, although the extent to which NO_3^- and other anions stabilized the dead-end complex showed considerable species variation. These Class II anions were distinguished by Milner-White & Watts (1971) from other inhibitory anions (Class III), which did not form a dead-end complex, and from acetate (Class I), which

was found to activate in the direction of phospho-creatine synthesis.

Concomitant with this work Thiem *et al.* (1972) reported effects of anions as inhibitor of the arginine kinases from the worm, *Sipunculus nudus*, from the mollusc, *Solen ensisensis*, (razor shell) and more briefly, from *Homarus vulgaris*. Although they showed NO_3^- ions to be inhibitory below 50 mM concentration their particular interest was in the effects of Cl^- ions at high concentrations, above 100 mM, where inhibition appeared to be mainly caused by structural disorganization.

The work reported below was designed to discover whether arginine kinases were inhibited by low concentrations of anions, 100 mM and less, in a similar manner to the creatine kinases. With two arginine kinases, the monomeric form from the lobster, *Homarus vulgaris*, and the dimeric form from the sea cucumber, *Holothuria forskali*, evidence has been presented to indicate that stabilization of a substrate dead-end complex could occur. However, the picture is complicated by additional protein-ion interactions.

Materials and Methods

ADP (disodium salt) and ATP (disodium salt) were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. Arginine solutions were made

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from arginine (free base) from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. The pH was adjusted with dilute acetic acid. Tris was from BDH Chemicals Ltd., Poole, Dorset, U.K. Bicine [*NN*-bis-(2-hydroxyethyl)glycine] was recrystallized as described by Kumudavalli *et al.* (1970) and was a gift from Dr. B. H. Moreland. Other compounds were as described by Anosike *et al.* (1975).

Enzymes

Arginine kinase from lobster, *Homarus vulgaris*, was prepared as described by Virden *et al.* (1965). The enzyme from sea cucumber, *Holothuria forskali*, was purified as described by Anosike *et al.* (1975).

Methods

Enzyme assays. For experiments on the effect of anions on the inhibition of the enzyme by the dead-end complex, the phosphate assay was used. Enzyme activity was measured by determining the acid-labile phosphoarginine formed in 10 min at 30°C in a total reaction volume of 0.4 ml as described by Virden & Watts (1966). This gave a reasonable estimate of initial velocity, as rate of product formation was a linear function of time under the conditions used.

The coupled enzyme assay, using pyruvate kinase and lactate dehydrogenase (Anosike *et al.*, 1975), was

used to investigate the effect of anions on the initial-velocity pattern of the forward reaction catalysed by arginine kinases from the two sources. All reactions were carried out in 50 mM-Tris-acetate buffer, pH 8.6. For the double-reciprocal plots the data were regressed by using an Olivetti desk computer with a program giving equal emphasis on all points.

Difference spectra. These were measured in a Cary 16S recording spectrophotometer by using split-path-length cells as described by Focant & Watts (1973).

Results

Inhibition of the forward reaction by anions alone

Concentrations of the linking enzymes used were high, so that their possible inhibition by anions under investigation would not be rate-limiting in the overall reaction. Before use, both lactate dehydrogenase and pyruvate kinase were dialysed against 50 mM-ammonium acetate in 5 mM-Bicine-NaOH buffer, pH 8.6, for 24 h with three changes of buffer to remove $(\text{NH}_4)_2\text{SO}_4$ present in the commercial preparations of these enzymes.

The effects of selected anions on the initial velocities of the forward reactions catalysed by the two arginine kinases are shown as double-reciprocal plots in Figs. 1 and 2. Under the conditions used the dead-end complex, arginine-enzyme-MgADP, will

Fig. 1. Effect of various anions on the forward reaction of lobster arginine kinase

(a) At 2 mM fixed arginine concentration: ●, +50 mM-ammonium acetate; ○, +50 mM-KCl; ▲, no addition (control); △, +50 mM- NaNO_2 ; ■, +50 mM- NaNO_3 . (b) At 2 mM fixed MgATP concentration: ●, +50 mM-ammonium acetate; ○, +50 mM-KCl; ▲, no addition; △, +50 mM- NaNO_2 ; ■, +50 mM- NaNO_3 . A 2.6 $\mu\text{g}/\text{ml}$ solution of enzyme was used for each reaction in the coupled assay at 30°C and pH 8.6.

progressively form as the reaction proceeds only if it is sufficiently stable that the MgADP is not utilized by the coupled assay. In fact, there was no evidence that this occurred with either enzyme. The anions were chosen because with the rabbit enzyme NO₃⁻ and NO₂⁻ had the greatest inhibitory effect; Cl⁻ was less inhibitory, whereas acetate acted as an activator and did not stabilize the dead-end complex. Fig. 1 shows for lobster arginine kinase that acetate and Cl⁻ activate, whereas NO₂⁻ and NO₃⁻ both inhibit non-competitively at 50mM concentration. Similar results were obtained with the *Holothuria* enzyme (Fig. 2). All the anions affected V_{max}. to some extent, but only NO₂⁻ and NO₃⁻ apparently altered K_m, raising that for ATP with the lobster enzyme and lowering that for arginine with the *Holothuria* enzyme (Table 1). Because of the complex nature of the effect found with NO₃⁻ (see below) it was considered that inhibitor constants calculated from these data in the absence of more extensive information might only prove misleading and, instead, the percentage effect on the initial velocity and the apparent K_m under defined conditions are compared in Table 1.

Fig. 3 shows a more detailed investigation of the effect of NO₃⁻ on the lobster enzyme. Extrapolation of the plots gives the point of intersection in the third quadrant. As the NO₃⁻ concentration is raised to 15mM both V_{max}. and the apparent K_m are progressively lowered. The data yield straight lines as Dixon (1953) plots which also intersect in the third quadrant, indicating mixed inhibition with respect to MgATP, from which a K_i value of 13mM was calculated.

Assuming that, as for creatine kinase, the catalytic reaction follows rapid-equilibrium random kinetics (Anosike *et al.*, 1975), K_s, K_i, K_s' and K_i' may be

determined in the manner outlined by Dixon & Webb (1964). These are the dissociation constants for the following reactions respectively:

K_s (≡ K_m) for MgATP is 0.25mM (Table 1). K_s', equal to K_s in the presence of saturating concentrations of

Fig. 2. Double-reciprocal plots showing the effects of some anions on the forward reaction of *Holothuria* arginine kinase at pH8.6 and 30°C

The MgATP concentration was fixed at 2mM. Δ, No addition; ●, +50mM-ammonium acetate; ○, +50mM-NaCl; △, +50mM-NaNO₂. For each assay 0.2μg of enzyme was used. Other conditions were as in Fig. 1.

Table 1. Effects of various anions on the initial velocity of the forward reactions catalysed by lobster and *Holothuria* arginine kinases

The percentages of inhibition or activation were measured at 2.0mM concentration of the fixed substrate. The data are derived from double-reciprocal plots similar to those in Figs. 1 and 2. Other details are given in the Results section.

Variable substrate	Anion (50mM)	Lobster arginine kinase			Holothuria arginine kinase		
		Activation (%)	Inhibition (%)	Apparent K _m (mM)	Activation (%)	Inhibition (%)	Apparent K _m (mM)
Arginine	None	—	—	0.11	—	—	0.80
	NO ₃ ⁻	—	79.0	0.13	—	35.0	0.37
	NO ₂ ⁻	—	33.0	0.14	—	29.0	0.44
	Acetate	40.0	—	0.24	36.8	—	0.90
	Cl ⁻	19.0	—	0.09	13.2	—	0.83
MgATP	None	—	—	0.25	—	—	—
	NO ₃ ⁻	—	70.0	0.56	—	—	—
	NO ₂ ⁻	—	21.0	0.50	—	—	—
	Acetate	37	—	0.29	—	—	—
	Cl ⁻	13.0	—	0.27	—	—	—

Fig. 3. Inhibition of the forward reaction of lobster arginine kinase by NO_3^- at pH 8.6 and 30°C

(a) The arginine concentration was fixed at 2 mm and the enzyme concentration was 2.6 $\mu g/ml$. ●, Control without NO_3^- ; ○, +5 mm- $NaNO_3$; ▲, +10 mm- $NaNO_3$; △, +15 mm- $NaNO_3$. (b) Determination of K_i' from a plot of $1/V_{max}$ against $[I]$ by using the data from (a). Other details are given in the Results section.

Fig. 4. Activation and inhibition of the forward reaction of *Holothuria* arginine kinase by NO_3^- at pH 8.6 and 30°C

The MgATP concentration was fixed at 2 mm and the enzyme concentration was 0.08 $\mu g/ml$. ●, Control without NO_3^- ; ○, +10 mm- $NaNO_3$; ▲, +25 mm- $NaNO_3$; △, +50 mm- $NaNO_3$; ■, +100 mm- $NaNO_3$.

NO_3^- , may be determined from a plot of K_s against the reciprocal of the NO_3^- concentration. By using the data from Fig. 3(a) a value of K_s' of 0.17 mm is obtained. K_i may then be calculated from the intercepts on the ordinate of Fig. 3(a) ($1/V_p$) from the relationship

$$\frac{1}{V_p} = \frac{1 + iK_s/K_i K_s'}{V}$$

For NO_3^- concentrations of 5, 10 and 15 mm K_i values of 8.2, 9.2 and 10.1 mm respectively are obtained. This shows fair agreement with the value of 13 mm obtained from the Dixon plot. K_i' may be calculated from the relationships $K_i \cdot K_s' = K_i' \cdot K_s$. For a mean value of K_i (from the four determinations) of 10.1 mm a value of K_i' of 6.9 mm is obtained.

K_i' may also be determined graphically from a plot of the reciprocal of V , at saturating substrate concentrations, against I . This is shown in Fig. 3(b) by using the data from Fig. 3(a) and gives a value for K_i' of 7.6 mm which is in reasonable agreement with that obtained by calculation.

The similarity of the values of K_i and K_i' and of K_s and K_s' indicates that the competitive element in the mixed inhibition is not very great. It should be noted that this analysis gives no information about whether the breakdown of EIS, as well as that of ES, contributes to the overall rate of catalysis.

With the *Holothuria* arginine kinase NO_3^- is found to be both an activator and an inhibitor when arginine is the variable substrate, depending on the concentrations of arginine and anion used

Fig. 5. Results from Fig. 4, presented as a Dixon (1953) plot

The arginine concentrations were: ●, 2mM; ○, 1mM; ▲, 0.5mM; △, 0.33mM; ■, 0.25mM; □, 0.2mM. For details see Fig. 4 and the Results section.

(Fig. 4). With 10mM- NO_3^- the plot shows activation that is purely competitive with arginine. If the Lineweaver-Burk kinetics for competitive inhibition may be applied to this system (Krüger-Thiemer, 1969) then a K_a of 12.5mM is obtained for the activation process. As the NO_3^- concentration is progressively raised the double-reciprocal plots become progressively more curved and the activation is overcome by non-competitive inhibition as a secondary process (Fig. 4). The curved nature of the plots preclude an analysis similar to that used for the lobster enzyme. Fig. 5 shows the same data presented as a Dixon (1953) plot. As with the lobster enzyme these plots can be extrapolated to intersect in the third quadrant, indicating that the inhibition process is complex with an overall K_i value of 48mM. This is confirmed by a plot of the reciprocals of the slopes from Fig. 4 against NO_3^- concentration, which was non-linear, concave upwards. A plot of $1/V_{\max}$ from Fig. 4 against NO_3^- concentration gave a straight line and a K_i' value for the inhibition process alone of 20mM, and indicates that the anion is binding at the catalytic site. Taking the value of K_s for arginine of 0.8mM (Table 1) an approximate value for K_s' of 0.33mM may be calculated.

Effect of anions on the inhibition of the forward reaction by MgADP

The phosphate assay was used for these experiments, since, as mentioned above, MgADP is a substrate for pyruvate kinase in the coupled enzyme assay. The final reaction volume of 0.4ml was made up of 0.1ml of 8mM-L-arginine, 0.1ml of 16mM-MgATP, 0.15ml of 50mM-Tris-acetate, pH8.6, plus MgADP and anion and 0.05ml of suitably diluted enzyme. In each assay, magnesium acetate was added to a concentration greater by 1mM than the total

Fig. 6. Effects of NO_3^- and MgADP separately and combined on the forward reaction of lobster arginine kinase at pH8.6 and 30°C

The arginine concentration was fixed at 2mM and the enzyme concentration was 1.3 $\mu\text{g}/\text{ml}$. The phosphate assay was used; ●, control, no additives; ○, +0.43mM-MgADP; ▲, +10mM- NaNO_3 ; △, +0.43mM-MgADP and 10mM- NaNO_3 .

concentrations of ATP and ADP, as suggested by Cleland (1967). This would result in the effective chelation of the ATP and most of the ADP.

Fig. 6 shows the effect of NO_3^- on the inhibition of the lobster enzyme by MgADP. This experiment was designed to test if this ion enhances inhibition of enzyme activity by stabilization of the dead-end complex, arginine-enzyme-MgADP.

The effect of the two modifiers together, compared with when they are present separately, is markedly dependent on the MgATP concentration. With $V_{\max}$ conditions the degree of inhibition is not significantly different from that by 10mM- NO_3^- alone. It is only when the MgATP concentration has been lowered to 0.33mM that the inhibition becomes more than additive and then only by 5.6%.

In a second series of experiments the MgADP concentration was held at 0.43mM and the NO_3^- concentration was raised to 50mM. The synergistic effect of MgADP plus NO_3^- was then 11.3% more than the sum of the two modifiers separately (Table 2). A series of other anions tested under these conditions all failed to show any significant enhancement of MgADP inhibition (Table 2), from which it may be inferred that only NO_3^- possibly acts to stabilize the dead-end complex. As a further check some of the

Table 2. *Experiment to determine the enhancement of inhibition of lobster arginine kinase at 30°C by MgADP plus various anions as compared with the inhibition caused by each separately*

Inhibition was measured in the direction of phosphoarginine synthesis. Other details are in the Results section.

Anion	Inhibition (%)				Enhancement of inhibition by MgADP in presence of anion (%)
	MgADP alone (0.43 mM)	Anion alone (50 mM)	Calc. for anion+ MgADP	Found for anion+ MgADP	
NO ₃ ⁻	21.4	53.8	63.7	75.0	+11.3
NO ₂ ⁻	21.4	21.1	38.0	32.2	-5.8
Cl ⁻	21.4	13.6	32.1	25	-7.1
Br ⁻	21.4	16.5	34.4	32.4	-2.0
I ⁻	21.4	27	42.6	31.3	-11.3
F ⁻	21.4	22	35.7	27	-11.7
SCN ⁻	21.4	24.7	40.8	33	-7.8
BO ₃ ⁻	19.0	5.9	23.8	26.8	+3.0
HCO ₂ ⁻	19.0	9.8	26.9	27.8	-0.9
SO ₄ ²⁻	19.0	50.0	59.5	54.5	-5.0

Table 3. *Experiment to determine the enhancement of inhibition of Holothuria arginine kinase at 30°C by MgADP plus various anions as compared with the inhibition caused by each separately*

Inhibition was measured in the direction of phosphoarginine synthesis. Other details are in the Results section.

Anion	Inhibition (%)				Enhancement of inhibition by MgADP in presence of anion (%)
	MgADP alone (0.43 mM)	Anion alone (50 mM)	Calc. for anion+ MgADP	Found for anion+ MgADP	
NO ₃ ⁻	29.3	33.3	52.9	66.7	13.8
NO ₂ ⁻	29.3	40.7	58.1	73.5	15.4
Cl ⁻	29.3	10.0	37	35.0	1.0
I ⁻	29.3	16.7	41.1	40.0	-1.1
Br ⁻	29.3	7.3	34.5	37.4	2.9
F ⁻	29.3	16.0	40.6	44.0	3.4
BO ₃ ⁻	29.3	26.0	47.7	45.3	-2.4
HCO ₂ ⁻	29.3	12.0	37.8	42.0	4.2

anions were also tested at 0°C instead of 30°C to investigate the possibility that the higher temperature, which is above the physiological range for this animal, might destabilize any complex formed. With 10mM-NO₃⁻ an enhancement of MgADP inhibition of 6.3% was obtained, which is very similar to that found at 30°C (Fig. 5). Other anions tested at 50mM gave the following percentage enhancements of inhibition: NO₂⁻ (13), Cl⁻ (4.8), Br⁻ (3.6) and BO₃⁻ (6.6), indicating that NO₂⁻ is now clearly stabilizing the dead-end complex and the other anions may be doing so to a very small extent.

Similar experiments on the stabilization of the dead-end complex were carried out on the *Holothuria* enzyme and are summarized in Table 3. These show that, again, only NO₃⁻ and NO₂⁻ cause significant enhancement of inhibition by MgADP for the formation of the dead-end complex to be inferred. NO₃⁻

and NO₂⁻ were also tested at 0°C and 10mM concentration and gave 4.8 and 10.4% enhancement of inhibition. Values for Cl⁻, Br⁻ and I⁻ at 50mM concentration and 0°C were almost identical with those found at 30°C (Table 3), so that lowering the temperature has very little effect with this enzyme.

Difference spectra measurements

The effect of NO₃⁻ on lobster arginine kinase was investigated by difference spectroscopy. The enzyme, 1.5mg/ml, in 0.1M-Tris-acetate buffer, pH 8.6, plus anion alone gave no difference spectrum. Addition of NO₃⁻ to enzyme plus MgATP (Fig. 7a) or enzyme plus arginine produced very large red shifts with a maximum around 300nm, which can most probably be ascribed to the ionization of tyrosine residues only. A similar result was obtained on addition of

Fig. 7. Effect of NO_3^- on the difference spectra of lobster arginine kinase in the presence of various substrate combinations in 0.1 M-Tris-acetate buffer, pH 8.6

Split-path-length cells were used with each half-cell of 4.5 mm path-length. The volume of solution in each half-cell was 1 ml before addition of NaNO_3 solution. The enzyme concentration was 0.75 mg/ml after mixing. —, Difference spectrum before adding NO_3^- ;, difference spectrum after the addition of $10 \mu\text{l}$ of 1 M- NaNO_3 to each compartment of both cells and remixing the sample cell. (a) Difference spectrum with 0.05 mM-MgATP; (b) difference spectrum with 5 mM-arginine + 0.05 mM-MgATP. Each division on the ordinate represents 0.001 extinction unit.

NO_3^- to enzyme plus arginine plus MgATP (Fig. 7b), to enzyme plus MgADP, to enzyme plus an equilibrium mixture of substrates or even to enzyme plus either MgIDP or MgAMP. Two different enzyme preparations were used and both gave similar results.

Discussion

The effects of anions on the arginine kinases reported here are undoubtedly more complex than those found with rabbit muscle creatine kinase by Milner-White & Watts (1971), although a common behaviour pattern is apparent. As pointed out by them it is not possible to maintain constant ionic strength in these experiments because of interactions with the system under investigation. Fortunately ionic-strength effects appear not to be a problem with these arginine kinases, as in a subsequent series of experiments using arginine analogues (E. O. Anosike, B. Moreland & D. C. Watts, unpublished work) a similar variation in ionic strength to that used here was found with some compounds to be without a significant effect on the system.

Both arginine kinases were activated in the forward reaction by acetate, showing that, as with creatine kinase, this behaves as a Class I anion. The finding that Cl^- also activated was surprising, since this anion was found by Milner-White & Watts (1971) effectively to stabilize the dead-end complex creatine-enzyme-MgADP. However, these authors did not investigate the effects of anions in the absence of MgADP by using the coupled assay system. It does seem that with anions that are relatively weak inhibitors, as in this arginine kinase system, the MgADP was effectively removed by the coupled assay system as fast as it was synthesized, so that in this respect the present work is not comparable. It may nevertheless be compared with the work of Thoai and his co-workers (Thiem *et al.*, 1972; Lacombe *et al.*, 1973) who found, using the coupled assay system, that both acetate and Cl^- were inhibitory. The difference in findings may be explained because Thoai's group used concentrations of Cl^- up to 0.4 M and of acetate up to 0.6 M. This suggests that a different phenomenon was being examined by them which may well be explained, as they proposed, by a general perturbation of protein structure.

Thiem *et al.* (1972) also found that the arginine kinases of *Solen* and *Sipunculus* were inhibited by concentrations of NO_3^- lower than 50 mM. The present work supports the generality of this finding, but indicates that complex kinetic relationships exist that may be characteristic of a particular species. With the dimeric *Holothuria* kinase NO_3^- has clear activating and inhibitory effects that may readily be distinguished. Inhibition is of the mixed type and may be presumed to occur at the active centre. Mixed inhibition was also found with the lobster enzyme. For both enzymes $K_s > K_i'$ and $K_i > K_i'$, indicating the unusual situation that the presence of the inhibitor promotes binding of the substrate and vice versa. It should be noted, however, that for *Holothuria* arginine kinase the substrate is MgATP and for the lobster kinase it is arginine. Thus if these enzymes

may be considered as homologous in their catalytic organization (see below) then NO_3^- may be inferred to bind in such a way as to influence both substrates even in the absence of a dead-end complex. This is in accord with the finding that NO_3^- will stabilize the enzyme-arginine-MgADP complex and also with the finding for the lobster enzyme that either substrate will promote the appearance of an enzyme- NO_3^- difference spectrum.

Of the range of anions tested only NO_3^- and NO_2^- clearly caused the enhancement of inhibition associated with stabilization of the dead-end complex, arginine-enzyme-MgADP. With the lobster enzyme, stabilization by NO_3^- could only be demonstrated convincingly by lowering the assay temperature from 30°C to 0°C. With the *Holothuria* enzyme, lowering the assay temperature conferred no obvious additional stability on the dead-end complex, which may perhaps be attributed to the difference in temperature stability of the monomeric and dimeric forms of the enzyme. For this enzyme NO_2^- appeared rather more effective than NO_3^- . The lesser efficacy of anions in stabilizing the lobster enzyme complex as compared with the *Holothuria* enzyme complex may also relate to the finding that the lobster enzyme shows a lower degree of positive co-operativity between substrates binding to the enzyme (Anosike *et al.*, 1975). Bearing in mind the observation that co-operativity between substrates for creatine kinase is associated with the substrates becoming more precisely orientated, more tightly bound and to some extent drawn together (Cohn *et al.*, 1971; Reed & Cohn, 1972; Reed *et al.*, 1972), this might be expected and provides support for the hypothesis that the role of the anion in this system is to mimic the transferable phosphoryl group in its planar transition state.

The difference spectra (Fig. 7) show a direct interaction of lobster arginine kinase with NO_3^- , apparently with a profound effect on the ionization of tyrosine residues. It is noteworthy that the anion does not produce a difference spectrum with the enzyme alone, but only with an ES complex. This suggests that there is an uncompetitive element to the mixed inhibition revealed by the kinetics. Since either substrate, or even other nucleotides such as IDP or AMP, facilitates production of the difference spectrum, it is reasonable to conclude that NO_3^- binds directly to the enzyme. However, the data do not exclude the possibility that NO_3^- can bind to the free enzyme and that formation of the difference spectrum is in some way promoted by the subsequent binding of a substrate or substrate analogue.

The clear involvement of tyrosine residues (Fig. 7) is of considerable interest in the light of the finding by Fattoum *et al.* (1971) that this enzyme is inactivated by iodination or nitration of a single tyrosine residue, and that enzyme so modified no longer gives a difference spectrum with either substrate (Roustan

et al., 1970). It has been suggested (Landon *et al.*, 1970) that this tyrosine residue is located in a helical region of the enzyme, a great part of which is exposed to the solvent. Nitration of this group produces considerable structural rearrangement, although no further changes occur on reaction of three additional tyrosine residues. The effect of NO_3^- reported here would fit in with these observations if binding in some way moved the tyrosine residue from a less ionizing to a more ionizing environment. It would not, of course, necessarily involve a direct interaction between NO_3^- and the tyrosine residue. Finally, this observation explains why a similar effect of NO_3^- is not found with rabbit muscle creatine kinase, since, as shown by Roustan *et al.* (1970), creatine kinase does not have a similarly accessible tyrosine residue.

In conclusion the present work demonstrates that two arginine kinases behave like the creatine kinases from a variety of species (see the introduction) in forming an anion-stabilized dead-end complex. This suggests that the general mechanism of catalysis is similar for both groups of enzymes and is in accord with the general hypothesis that all the guanidine kinases have a common evolutionary origin (Watts, 1971, 1973). As compared with that found with the creatine kinases, the stabilizing efficiency of the anions was particularly weak, with only those possessing a close structural similarity to a planar phosphoryl group, NO_3^- and NO_2^- , being significantly effective. Whether or not this relates to the evolutionary change in the enzyme structure to accommodate a different guanidine substrate may become clear when the investigation has been extended to a wider range of arginine kinases and other guanidine kinases. However, the effect does seem to relate to the extent of the co-operative interactions between substrates at one catalytic site. This is not very strong for the *Holothuria* enzyme and is almost non-existent for the lobster kinase (Anosike *et al.*, 1975). The monomer lobster enzyme is also much more sensitive to denaturing agents than the dimeric *Holothuria* enzyme (Anosike *et al.*, 1975) and this offers a reasonable explanation of why stabilization of the dead-end complex by NO_3^- only becomes significant when the temperature is lowered to 0°C. The other anion effects reported may be considered as secondary to structural changes in other parts of the enzyme structure during evolution. The apparently essential tyrosine residue of the lobster enzyme (Fattoum *et al.*, 1971) is probably present but masked in creatine kinase (Watts, 1973). Binding of free NO_3^- to the lobster enzyme causes the tyrosine residue to become exposed to a more ionizing environment, and this may relate both to the observed inhibition and the lesser stability of the monomer. The effects of low concentrations of anions on enzyme activity under conditions where the reaction product, ADP, is removed from the system have not been

investigated for creatine kinase. The activation of these arginine kinases by Cl^- (Table 1), and of the *Holothuria* enzyme by NO_3^- at low concentrations, (Fig. 4), may involve interactions at the acetate-binding site. This is a distinct entity in creatine kinase to which the binding of certain anions causes activation (Watts, 1973). The same site now appears to be a feature of arginine kinases as well. Whether the activation by Cl^- , an inhibitor of the creatine kinases, also involves binding at this site has still to be determined. The possible role of this site in enzyme regulation, which, like the catalytic site and subunit interacting site seems to have been strongly conserved in evolution, will be a rewarding area for future investigation.

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