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SPECTROPHOTOMETRIC INVESTIGATIONS OF THE INTERACTION OF NATIVE AND CHEMICALLY MODIFIED ATP:GUANIDINOPHOSPHOTRANSFERASES WITH THEIR SUBSTRATES

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SUMMARY

The nucleotide dissociation constants and the number of binding sites for different phosphagen kinases have been determined. It is shown that the spectral patterns observed are similar for all the enzymes studied and may result from chromophoric perturbation of nucleotides as well as from specific alterations of the micro-environment of the protein-binding sites. The results obtained in the study of the binding of several nucleotide analogs to arginine kinase indicate that the formation of active nucleotide–enzyme complexes requires the presence of the γ- and β-phosphoryl groups as well as the 6-amino and N-1 groups in the purine ring.

The spectral patterns observed with the native enzymes have been compared with those of chemically modified arginine and creatine kinases. Dansylation of the essential lysyl residues alters the binding properties of ADP–Mg^{2+} but has no effect on ATP–Mg^{2+}, and no enzyme–substrate complex is observed between 1-dimethylamino-5-naphthalenesulphonyl(DNS)-arginine kinase and L-arginine. Carbethoxylation of the active histidine residue in both enzymes causes no modification of the difference spectra though no transphosphorylation occurs. Nitration of one tyrosine residue in arginine kinase abolishes the interaction between this enzyme and its substrates, and whereas the modification of lysyl and histidyl groups does not result in any conformational changes, tyrosine nitration produces an important decrease in the Cotton effect at 233 mμ.

INTRODUCTION

A differential spectroscopic method was used in previous work to study the specific interaction of nucleotide and guanidine substrates with native arginine- and creatine kinases[1].

In the first part of the present communication we extend these investigations to taurocyamine and lombricine kinases in order to obtain more information about the

Abbreviation: DNS-, 1-dimethylamino-5-naphthalenesulphonyl- group.

mechanism of the interaction of the phosphagen kinases with their substrates. In the same way the binding of nucleotide analogs was studied in order to relate the spectral changes observed to the structure of the nucleotide substrates.

In the second part the involvement of some active residues in the formation of arginine kinase– and creatine kinase–substrate complexes is investigated by means of ultraviolet difference spectroscopy. Previous reports from our laboratory have indeed shown that these phosphagen kinases possess cysteinyl[2], histidyl[3] and lysyl[4] residues at their active sites but their precise function in the catalytic activity is not yet known. The data described below were obtained by studying the interaction of guanidine and nucleotide substrates with enzymes chemically and specifically modified at their histidyl and lysyl residues. The spectral patterns have been compared with those currently observed with the native phosphagen kinases. In addition, the role of a tyrosyl residue, the essential feature of which has been recently demonstrated in arginine kinase[5], was also investigated.

MATERIAL AND METHODS

Chemicals, substrates and analogs

Dansyl chloride is a product of the Pierce Chemical Co. and is used without purification. Diethylpyrocarbonate is provided by Carlo Erba. Tetranitromethane is obtained from Aldrich Chemical Co. $K_2S_4O_6$ is a Merck product, 5,5'-dithiobis(2-nitrobenzoic acid) is a Pierce Chemical reagent and dithiothreitol is provided by NBC. All the other products used are of the best analytical grade.

Adenine and adenosine were obtained from Hoffman La Roche; all the other nucleotides (A grade) were purchased from Calbiochem. Their concentration was determined from their molar extinction[6]. 5-Phosphoryl ribose 1-pyrophosphate was a product from Sigma Chemical Co. All the nucleotide solutions were adjusted to pH 7 by addition of NaOH (0.1 M).

Phosphagen kinases

Crystalline creatine kinase (EC 2.7.3.2) (specific activity 70) was obtained according to the method of KUBY[7] (Procedure B). Arginine kinase (EC 2.7.3.3) from lobster muscle (specific activity 230) was prepared according to the method of DER TERROSSIAN[8]. Lombricine kinase (EC 2.7.3.5) from *Lumbricus terrestris* muscle and taurocyamine kinase (EC 2.7.3.4) from *Arenicola marina* muscle were prepared as previously described[9], with specific activities of 90 and 170, respectively.

Protein concentration and molecular weight

Protein concentration was calculated from the absorbance at 280 mμ on the assumption that $E^{1\%}_{1cm}$ arginine kinase = 7.35 (ref. 10) and $E^{1\%}_{1cm}$ creatine kinase = 8.8 (ref. 11), or according to the method of WARBURG AND CHRISTIAN[12]. Molecular weights of 43 000 (arginine kinase) (ref. 13), 81 000 (creatine kinase) (ref. 14), 74 000 (lombricine kinase) (ref. 15) and 81 000 (taurocyamine kinase) (ref. 15) are used to calculate molar concentrations.

Physical measurements

Ultraviolet difference spectra were measured with a Cary model 15 spectro-

photometer supplied with thermostated compartments. Matched pair quartz cells of 1-ml capacity and 0.4375-cm light path were used. In order to obtain the base line the cells were filled with 0.9 ml of enzyme solution in one compartment and 0.9 ml of buffer solution in the other; they were scanned against one another from 330 to 235 mμ using the 0–0.1 slide wire.

The reaction was started by adding (with a Hamilton microsyringe) 5–100 μl ligand solution to the enzyme sample compartment as well as to the buffer reference compartment. The dilution effect was corrected by adding an identical volume of distilled water to the two other compartments.

The binding constants and the number of catalytic sites were determined as previously described[1]. Values for maximal differential absorption at fixed wavelengths, extrapolated to infinite substrate concentration, were used to calculate the molar absorption $\Delta\varepsilon_M$ of the studied ligand–protein complexes.

Optical rotatory dispersion measurements were performed with a Fica type Spectropol I spectropolarimeter using 0.1 M Tris–acetate buffer, (pH 7.5) and a 1-cm lightpath cell.

Preparation of chemically modified phosphagen kinases

(a) DNS labelling. 10 mg/ml of native arginine kinase or *S*-sulfenyl sulfonate creatine kinase[4] are dansylated in 0.05 M sodium bicarbonate buffer, (pH 8.0–8.5) as described in a previous paper[4]. The inhibited dye-labelled proteins are purified by molecular sieving on Sephadex G-25 (ref. 4). Creatine kinase –SH groups are recovered by exhaustive dialysis against 1 mM dithiothreitol–0.01 M phosphate buffer (pH 8). Every protein sample is tested for the number of DNS groups present by measuring its absorption at 335 mμ (ref. 16) and for the number of –SH groups by means of the 5,5′-dithiobis(2-nitrobenzoic acid) method[2]. Remaining activity is also determined[17].

(b) Protein carbethoxylation. 10–12 mg/ml of phosphagen kinases in 0.05 M phosphate buffer (pH 6.1) are allowed to react with diethylpyrocarbonate under the conditions described in Figs. 2 and 3, until 90–100% inhibition is obtained. The preparations are freed from excess of reagent by filtration on Sephadex G-25 equilibrated with 0.01 M Tris–acetate buffer (pH 7.0). The number of carbethoxylated imidazole groups is calculated from their specific difference absorption at 240 mμ (refs. 3, 18).

(c) Arginine kinase nitration. Nitration of arginine kinase with tetranitromethane is performed in 0.05 M Tris–acetate buffer, (pH 8.0) after masking the –SH groups with $K_2S_4O_6$. This chemical modification is reported in more detail in another paper[5].

RESULTS

(A) Binding of nucleotide substrates and analogs to native phosphagen kinases

Interaction of lombricine and taurocyamine kinases with nucleotide substrates ATP–Mg^{2+} or ADP–Mg^{2+}. The difference spectrum at neutral pH due to ADP–Mg^{2+} interaction with lombricine and taurocyamine kinases shows two typical positive peaks at 290 and 278 mμ, and a negative one at 254 mμ (Fig. 1A); in contrast, the binding of ATP–Mg^{2+} produces only a minimum at 254 mμ (Fig. 1B).

Stoichiometry. The different phosphagen kinases studied exhibit very similar

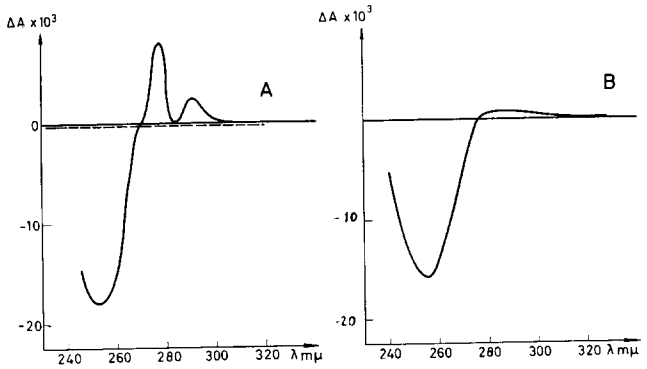

Fig. 1. Nucleotide–lombricine kinase complexes difference spectra. 0.1 M Tris–acetate buffer (pH 8.5). Temp., 20°. 0.4375-cm light path cells. Enzyme, 19 μM. A. Effect of lombricine (9 mM) (— — —) and ADP = magnesium acetate (90 μM) (———). B. Effect of ATP = magnesium acetate (0.18 mM).

dissociation constant values for nucleotide substrates (Table I and Fig. 2), but ADP–Mg^{2+} has always a higher binding affinity for the enzymes than ATP–Mg^{2+}.

The number of binding sites calculated according to the method used for creatine and arginine kinases[1] is equal to one for lombricine kinase and two for taurocyamine kinase*. These data are confirmed by those obtained using the molar extinction at 254 mμ of the enzyme–ADP–Mg^{2+} complex in the calculation of the number of sites; indeed, the magnitude of the peak at this wavelength increases proportionately with the number of sites (Table I).

Interaction of arginine kinase with nucleotide analogs. Various derivatives including adenosine, adenine, AMP, 5-phosphoryl ribose 1-pyrophosphate, pyrophosphate and tripolyphosphate have been studied and compared with the spectral changes observed with ATP and ADP (Fig. 3A).

The binding of adenosine, adenine and AMP (Fig. 3A) produces a positive difference spectrum in the 259–263-mμ region, but no spectral change occurred in the presence of other compounds. In contrast, with nucleotide diphosphate (CDP, IDP) and nucleotide triphosphate (CTP, UTP, GTP), a negative hypochromic difference

TABLE I

DETERMINATION OF BINDING CONSTANTS (K_s), NUMBER OF SITES (n) AND MOLAR EXTINCTION ($\Delta\varepsilon_m$) OF PHOSPHAGEN KINASES–NUCLEOTIDE COMPLEXES

Enzyme	n	*ADP–Mg^{2+}*		*ATP–Mg^{2+}*
		$\Delta\varepsilon_m$*	K_s *(mM)*	K_s *(mM)*
Arginine kinase	1	5 000	0.065	0.40
Creatine kinase	2	9 000	0.043	0.30
Lombricine kinase	1	4 500	0.10	0.40
Taurocyamine kinase	2	10 000	0.11	0.30

* In Table I the data are compared with those of arginine and creatine kinases found previously[1].

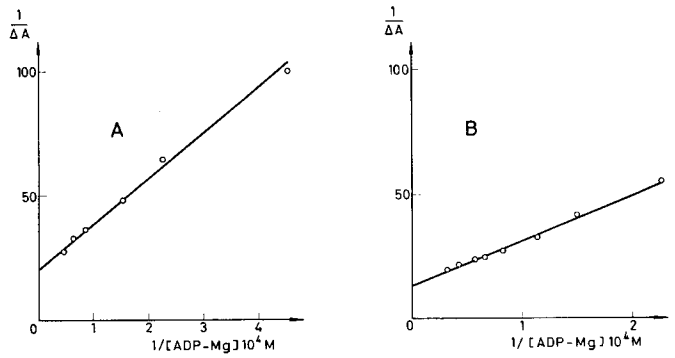

Fig. 2. Lineweaver–Burk representation for ADP–Mg^{2+} binding to lombricine kinase and taurocyamine kinase. 0.1 M Tris–acetate buffer, (pH 7.2). Temp., 20°. A. Lombricine kinase (21 μM). B. Taurocyamine kinase (18 μM).

TABLE II

VARIOUS NUCLEOTIDE BINDING CONSTANTS FOR ARGININE KINASE

Substrate:	*AMP*	*Adenosine*	*CDP*	*CTP*	*IDP*	*UTP*
K_s (mM)	0.6	1	0.3	1.5	2	5

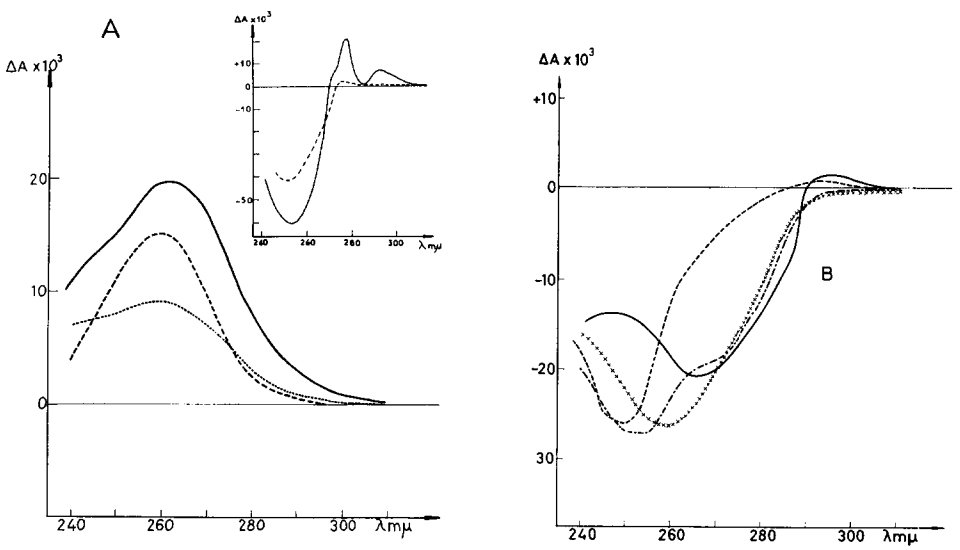

Fig. 3. Nucleotide analog–arginine kinase complexes difference spectra. A. Effect of adenine compounds in 0.1 M Tris–acetate buffer (pH 7.15). Temp., 25°. 0.4375-cm light path cells. ———, AMP (0.17 mM), enzyme (40 μM); — — —, adenosine (0.18 mM), enzyme (38 μM); . . . . ., adenine (0.2 mM), enzyme (38 μM). Inset: arginine kinase (36 μM). ———, effect of ADP (0.11 mM) in 0.1 M Tris–acetate buffer (pH 7.15). — — —, effect of ATP (0.34 mM) in 0.1 M Tris–acetate buffer (pH 8.5). B. Effect of nucleotide analogs. Temp., 20°. 0.1 M Tris–acetate buffer. 0.4375-cm light path cells. ———, CDP (0.42 mM), enzyme (39 μM) (pH 7.15); — — —, IDP (0.35 mM), enzyme (43 μM) (pH 7.15); × × ×, UTP (0.35 mM), enzyme (42 μM) (pH 8.5). — . —, GTP (0.35 mM), enzyme (42 μM) (pH 8.5).

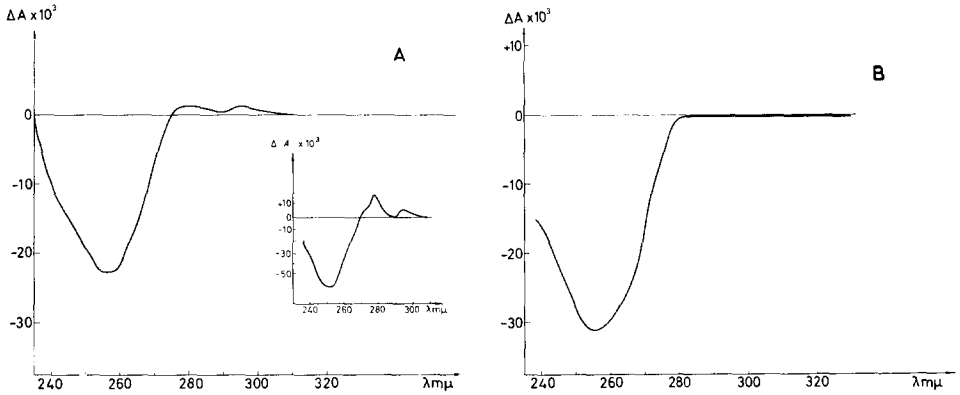

Fig. 4. Interaction of nucleotide substrates with DNS-arginine kinase. Modified arginine kinase (27 μM) 85% inhibited (0.7 mole of DNS per mole of enzyme) in 0.05 M Tris–acetate buffer (pH 8.5). Temp., 20°. 0.4375-cm light path cells. A. Effect of ADP = magnesium acetate (0.11 mM). Inset: effect of ADP = magnesium acetate (0.11 mM) on native enzyme (38 μM). B. Effect of ATP = magnesium acetate (0.22 mM).

spectrum occurs, as illustrated in Fig. 3B; the corresponding dissociation constants were evaluated (Table II).

(B) Binding of substrates to chemically modified phosphagen kinases

Lysine-modified phosphagen kinases. Fig. 4B shows that the inhibition of arginine kinase (as well as of creatine kinase) by dansylation of one essential lysine side chain does not alter the difference spectra obtained when ATP–Mg^{2+} binds to the enzymes. In contrast, when ADP–Mg^{2+} is used as substrate, the difference spectra obtained are strongly modified in that the two positive bands at 278 and 290 mμ have disappeared, whereas the minimum peak at 254 mμ remains unchanged (Fig. 4A). DNS-arginine kinase possessing all its –SH groups does not interact with L-arginine since it fails to give the characteristic difference spectrum of tyrosine which occurs by binding of the guanidine substrate to the native enzyme[1]. Optical rotatory dispersion measurements on the DNS-labelled proteins show no modification of the α helix content (Fig. 7).

Histidine-modified phosphagen kinases. Arginine kinase inhibited by reaction with diethylpyrocarbonate produces the same typical difference spectra as the native enzyme when it binds ATP–Mg^{2+}, ADP–Mg^{2+} and arginine, the two latter substrates being bound to a lesser extent (Fig. 5). When ATP–Mg^{2+} and L-arginine are added simultaneously, a differential spectrum is obtained which corresponds to the individual binding of the two substrates, without the appearance of 278–294-mμ absorption bands, which are characteristic of the formation of ADP–Mg^{2+}, as in the case of the native enzyme (Fig. 5).

The same results have been obtained with diethyl pyrocarbonate-inhibited creatine kinase (Fig. 6).

No structural modification of the carbethoxylated protein has been detected by optical rotatory dispersion measurement (Fig. 7).

Arginine kinase with a nitrated tyrosine residue. Arginine kinase completely inhibited by nitration of one tyrosine residue, even after total reduction of its masked

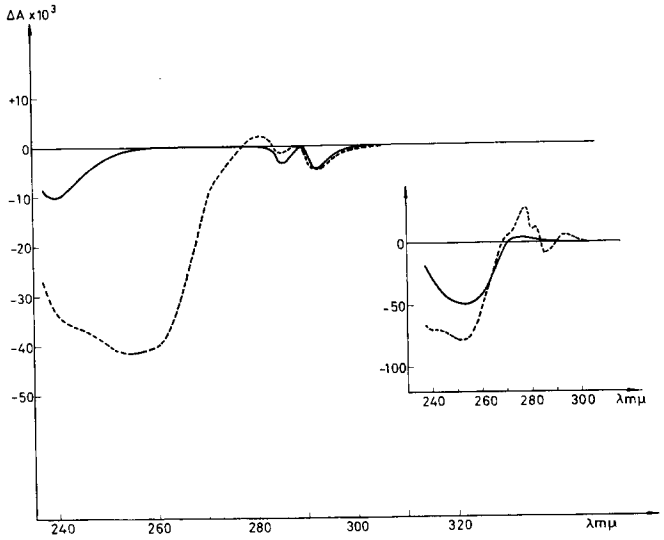

Fig. 5. Interaction of arginine and ATP–Mg^{2+} with carbethoxylated arginine kinase. The complete inhibition is obtained by treatment of 20 mg protein, for 10 min, in 2 ml 0.05 M phosphate buffer (pH 6.3) with 1 or 2 μl of 3 M diethylpyrocarbonate (1.4–2.5 moles of histidine per mole enzyme are modified). Difference spectra: enzyme (35 μM) in 0.05 M Tris–acetate buffer (pH 8.5). Temp., 20°. 0.4375-cm light path cells. ———, effect of L-arginine (4.4 mM); — — —, effect of L-arginine (4.4 mM) + ATP = magnesium acetate (0.21 mM). Inset: native arginine kinase (35 μM). ———, effect of ATP = magnesium acetate (0.4 mM); — — —, effect of ATP = magnesium acetate (0.4 mM) + L-arginine (2.2 mM).

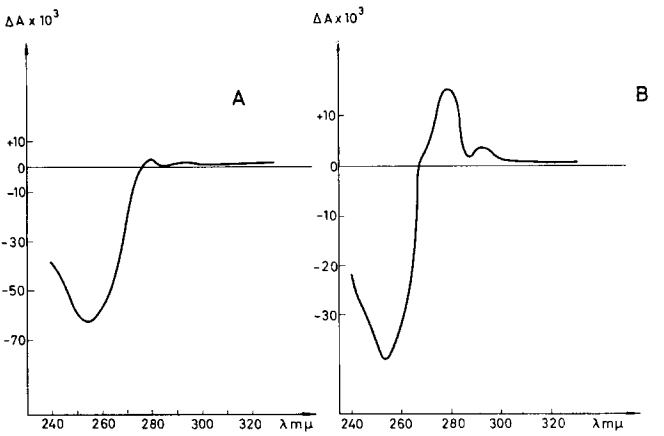

Fig. 6. Interaction of substrates with carbethoxylated creatine kinase. 90% inhibited creatine kinase is obtained by treatment of 25 mg enzyme, for 2 min, in 2 ml 0.05 M phosphate buffer (pH 6.1) with 3 μl of 1 M diethylpyrocarbonate (3 moles of histidine per mole enzyme are modified). Difference spectra: A. Effect of ATP–Mg^{2+} + creatine in 0.05 M Tris–acetate buffer (pH 8.5); Temp., 20°; 0.4375-cm light path cells; enzyme (20 μM); ATP = magnesium acetate (0.44 mM) + creatine (11 mM). B. Effect of ADP–Mg^{2+} in 0.01 M Tris–acetate buffer (pH 7.0); enzyme (25 μM); ADP = magnesium acetate (0.22 mM).

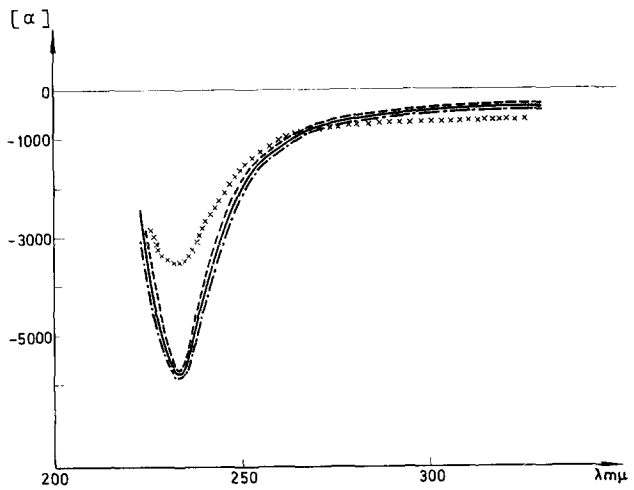

Fig. 7. Ultraviolet optical rotatory dispersion spectra of chemically modified arginine kinase in 0.1 M Tris–acetate buffer (pH 7.5). ———, native enzyme; — — —, DNS-enzyme. — · —, carbethoxylated enzyme. × × ×, mononitroenzyme.

–SH groups, does not show any change in ultraviolet absorption whether nucleotides or arginine are used as substrates. Fig. 6 shows that mononitroarginine kinase exhibits a decrease in the Cotton effect value at 233 mμ. The value $[\alpha]_{233\ m\mu} = -6000°$ for the native enzyme is reduced to $-3600°$ in the mononitroenzyme.

DISCUSSION

The present studies performed on native taurocyamine and lombricine kinases confirm our earlier findings describing the spectral changes which occur during the formation of phosphagen kinase–nucleotide complexes[1]. All the data obtained point out the same specific interaction of the nucleotide substrates with different phosphokinases; the spectral changes which have been observed are consistent with the generalisation that structural modification of the nucleotide chromophore as well as changes in the protein conformation occur during the enzyme–nucleotide complex formation. The metal ion Mg^{2+} would not seem to affect the arginine kinase–nucleo-

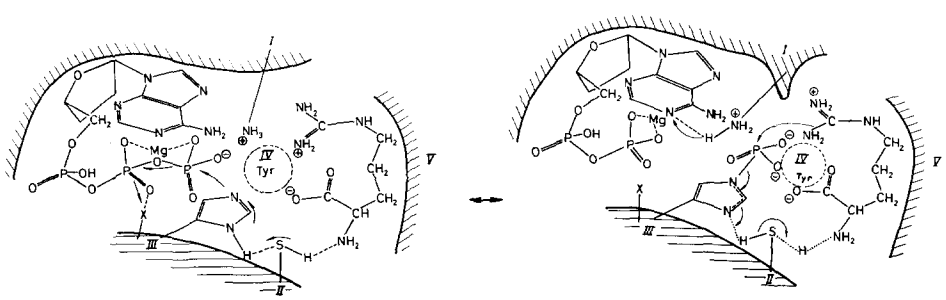

Fig. 8. Schematic representation of the working active site of arginine kinase. Sub-site I, lysyl residue; Sub-site II, cysteinyl residue; Sub-site III, histidyl residue; Sub-site IV, tyrosyl residue; Sub-site V, hydrophobic site for L-arginine binding.

tide interactions since the spectral patterns, as well as the binding constants, are the same with or without Mg^{2+}. A structural modification of the ATP or ADP adenine ring induces a hypochromic effect at 254 mμ; ADP–Mg^{2+} produces a specific red shift in the 270–280-mμ region; this effect may be explained by the protonation of N-1 of the 6-aminopurine ring[19–21]. The lack of protonation of the nucleotide ATP–Mg^{2+} may be explained by the proximity of the γ-phosphoryl group to the ε-NH_3^+ function of the lysine residue which is supposed to be responsible for the protonation of ADP as will be shown below; the close proximity of a negative charge and the formation of an ionic bond should prevent the protonation of the adenine ring in the ATP–Mg^{2+}–enzyme complex. On the other hand, a micro-environmental change in the subsequent protein structure related to the binding of ADP–Mg^{2+} is pointed out by a positive peak in the tryptophan chromophore region[22]. This localised conformational change is not observed when ATP–Mg^{2+} is the substrate.

Additional evidence for the relationship between these enzymes is also provided by the correlation which exists between the magnitude of the molar absorption spectra and the number of the nucleotide binding sites. The number of nucleotide binding sites found by the spectrophotometric method provides a suitable criterion for differentiation of the phosphagen kinases into two groups of enzymes; one includes taurocyamine and creatine kinases with two binding sites and the other concerns arginine and lombricine kinases with only one binding site.

In addition arginine kinase is the only enzyme which gives a characteristic difference spectrum upon binding its guanidine substrate[1]; in contrast, the interaction of creatine kinase[1], as well as lombricine and taurocyamine kinases, with their respective guanidine substrates fails to give any spectral change.

The spectral changes caused by the formation of complexes between arginine kinase and adenine compounds (adenine, AMP, adenosine) are different from those obtained with ADP and ATP and seem to prove the participation of the γ- and β-phosphate groups in the formation of the active complexes.

The relatively high affinity of CDP for arginine kinase compared with that found for the other nucleotide analogs also illustrates the importance of the presence of both the 6-amino group and the N-1 in the purine or pyrimidine ring.

Finally, it is of particular interest to note that the identical nucleotide–enzyme difference spectra obtained with the phosphagen kinases we studied may reflect the presence of similar nucleotide binding sites in this class of enzymes. Such nucleotide binding sites might have been highly selected during the course of evolution and preserved, at least in part, in other nucleotide related enzymes. Indeed, the nucleotide–enzyme complexes studied with alcohol dehydrogenase[23], glutamate dehydrogenase[24,25] and ribonuclease[21] exhibit difference spectra bearing a strong analogy to those we found in the case of the guanidinophosphotransferases.

The spectrophotometric studies performed with phosphagen kinases chemically modified at their essential residues may contribute to a better understanding of the role of these groups in the enzymic reaction.

(1) The results obtained with dansylated arginine- and creatine kinases show the direct involvement of the ε-NH_2 group of lysine in the protonation of nitrogen in the purine ring of ADP–Mg^{2+}; the implication of such a residue as arginine or lysine in the acid form has previously been envisaged by FISHER *et al.*[20] for liver alcohol dehydrogenase and by DEAVIN *et al.*[21] for bovine pancreatic ribonuclease. In the case of

arginine kinase, and probably of creatine kinase[26], this ε-NH_2 lysine side chain may be in close proximity to the essential –SH group, since interaction of arginine with the dansylated former enzyme fails to give the expected difference spectrum. The absence of a conformational change observed by means of optical rotatory dispersion measurements on the dansylated proteins suggests that the modified lysine residues in the two phosphagen kinases are possible specific ADP-binding sites.

(2) The spectral changes recorded during the interaction of nucleotide and guanidine substrates with the histidine-modified enzymes suggest that the imidazole group must be involved in the catalytic process, since binding of the two substrates occurs without phosphoryl group transfer. The mechanism of the phosphagen kinase-catalyzed reactions may be of the "ping-pong" or sequential type[27–30]; histidine would be associated with the reversible transfer of the labile phosphoryl group. The catalytic function of histidine is underlined by the lack of conformational change in the carbethoxylated proteins, as measured by the optical rotatory dispersion method.

(3) The most striking result observed when one tyrosine residue is nitrated in arginine kinase is the abolition of the spectral changes usually obtained by interaction of the nucleotides or L-arginine with the native enzyme. In contrast with the other chemical group modifications reported above, however, it was found that nitration was accompanied by conformational changes in the protein structure, as detected by a large decrease in the Cotton effect value at 233 mμ. These findings, together with other more detailed results[5], suggest that one tyrosine residue is directly related to the active conformation of arginine kinase.

On the basis of the spectrophotometric[1] and inhibition kinetic data[1–5] we have currently obtained for arginine kinase, a schematic representation of the active site is shown in Fig. 8, which should indicate the respective functions of the essential histidyl, cysteinyl, lysyl and tyrosyl residues in the supposed mechanism of action of the enzyme.

In Sub-site I, the lysyl residue is thought to be in the cationic form NH_3^+, which accounts for the protonation of N-1 of the purine ring in the enzyme–ADP complex and which is probably neutralized by the γ-phosphoryl group of ATP in the enzyme–ATP complex; the structure of the former complex should be different from that of the latter.

In Sub-site II, the essential pH-independent thiol group acts as a hydrogen donor for the α-NH_2 group of arginine, which is assumed to behave as a hydrogen acceptor on the basis of the crystallographic studies reported by RAMACHANDRAN[31]. The assumption that guanidine substrate interacts by means of its α-NH_2 group with the –SH group is based on the facts that not only are the difference spectra obtained with L-amino acids (isoleucine, valine, *etc...*) or –SH reagents (iodoacetamide, *N*-ethylmaleimide) (ref. 1 and results to be published) similar to those occurring with L-arginine, but also that the magnitude of the difference spectrum induced by total inhibition with –SH reagents is equal to that produced by the enzyme–arginine complex (results to be published).

In Sub-site III, a histidyl residue in close proximity to the thiol group acts as a general acid–base catalyst aiding the proton transfer from the –SH to the α-NH_2 group of arginine and catalyses the splitting of the terminal bond of ATP with the probable formation of an intermediary imidazyl phosphate (ref. 28 and results to be published). This assumption is substantiated by the fact that carbethoxyarginine

kinase catalyses neither the phosphoryl transfer reaction nor the partial isotope exchange between ATP and ADP (results to be published), though the enzyme still binds the substrates.

In Sub-site IV, the tyrosyl residue occupies such a suitable position in the active site that it maintains the components of this active site in the three-dimensional conformation required for efficient and specific catalysis.

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