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CONFORMATIONAL FLEXIBILITY AND STRUCTURE OF CREATINE KINASE

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The structural flexibility of creatine kinase has been investigated with the covalent hydrophobic probe 2-[4′-(2″-iodoacetamido) phenyl] aminonaphthalene-6-sulfonic acid (IAANS) which reacts at vastly different rates with the two subunits to give a protein conjugate with fluorescence characteristic of reaction with a site in a hydrophobic cleft. Binding of purine nucleotides greatly enhances the probe fluorescence while pyrimidine nucleotides quench the fluorescence. Small anions bind to nucleotide-free creatine kinase near the location of the transferable phosphoryl group and quench both the IAANS fluorescence of modified creatine kinase and the tryptophan fluorescence of native creatine kinase. Chloride and nitrate noncompetitively inhibit MgADP binding both with and without creatine. Fluorescence energy transfer demonstrates that the active sites of creatine kinase are well separated and become further apart after the nucleotide-induced conformational change.

INTRODUCTION

Creatine kinase is composed of two apparently identical subunits of axial ratio 4.4 lying side-by-side (1). A conformational change of small magnitude has been proposed on binding of nucleotides (2, 3). Two reactive thiol residues have been identified in creatine kinase (4) and their proximity to the active site demonstrated (5). Location of the thiols near the active site suggests that reaction with a sulfhydryl-directed covalent hydrophobic probe such as IAANS[1] (R. P. Haugland, unpublished observations) with environment-sensitive fluorescence characteristics might insert a reporter group to follow the conformational flexibility of creatine kinase. This paper reports on the nucleotide-induced conformational change, a new conformational change caused by anion binding, a different view of the so-called "anion-stabilized dead end complex," a difference in reactivity of the two subunits with IAANS, and the distance between the active sites of the two subunits.

[1] Abbreviations: 1,8-ANS, 8-anilinonaphthalene-1-sulfonic acid; IAANS, 2-[4′(2″-iodoacetamido) phenyl] aminonaphthalene-6-sulfonic acid; K TES, tris(hydroxymethyl)methylaminoethanesulfonic acid, potassium salt.

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METHODS

The methods used for protein modification, titrations with anions and nucleotides, and fluorescence measurements will be described in detail elsewhere, as will the synthesis of IAANS and the two fluorescence energy acceptors I and II. Rabbit skeletal muscle creatine kinase was obtained from Sigma Chemical Co. (St. Louis, Mo.) as were all nucleotides used.

RESULTS

Reaction of creatine kinase with the covalent hydrophobic probe IAANS occurs on the time scale of less than 2 min for the first subunit and several hours for the second subunit (Fig. 1). The probable sites of reaction are the cysteine residues located near the active sites (4). The fluorescence of the probe when bound to creatine kinase is enhanced about 30-fold above the fluorescence of IAANS reacted with 2-mercaptoethanol in water. This demonstrates that reaction with the protein leaves the chromophore in a hydrophobic cavity in which normal aqueous deactivation of the excited state is inhibited (6). Despite the loss in catalytic activity, all four substrates still bind to the modified enzyme.

Binding of nucleotides to IAANS creatine kinase results in a conformational change that is reflected in large changes in the probe fluorescence (Fig. 2). It was found that purine nucleotides enhance the fluorescence by as much as 213% while pyrimidine nucleotides quench the fluorescence by up to 54%. The fluorescence enhancement caused by ADP was used to demonstrate that the biphasic rate of reaction of IAANS with creatine kinase observed in Fig. 1 corresponds to reaction of identically situated residues on the two subunits. When the solutions in Fig. 1 were made 2.5 mM in ADP and the enhanced fluorescence plotted against the initial fluorescence without ADP, a linear plot resulted (not shown) which demonstrated that the probe fluorescence was enhanced identically at each site by ADP and that the conformational change responsible for fluorescence enhancement is identical on the two subunits.

The large fluorescence change caused by binding of nucleotides was used to calculate the apparent nucleotide dissociation constants from IAANS creatine kinase at 10°C and pH 7. As seen in Table I, the four purine diphosphates, ADP, GDP, IDP, and XDP are bound equally well by the enzyme, but the monophosphates are bound even more strongly and the triphosphate bound more weakly. The pyrimidine diphosphates UDP and CDP are bound an order of magnitude weaker than ADP and cause a fluorescence change in the opposite direction. The conformational change responsible for fluorescence enhancement is caused by the purine portion of the molecule since adenosine, although only weakly bound, is effective in enhancing the IAANS fluorescence. Despite being equally well bound, the extrapolated fluorescence enhancement at infinite nucleotide concentration for the purine diphosphates shows nucleotide specificity. This is reflected in the spectra in Fig. 2 taken at nearly saturating nucelotide concentrations. ATP and ADP cause the same fluorescence enhancement and, by inference, the same conformational change around the probe to bring the enzyme to an active state most suitable for reaction.

The effect of substrates and modifiers on nucleotide binding is seen in Table II. Most significant is the effect of Mg^{2+}, a catalytic activator, which has little effect on AMP and ADP binding but strongly inhibits ATP binding. Creatine slightly weakens ADP binding

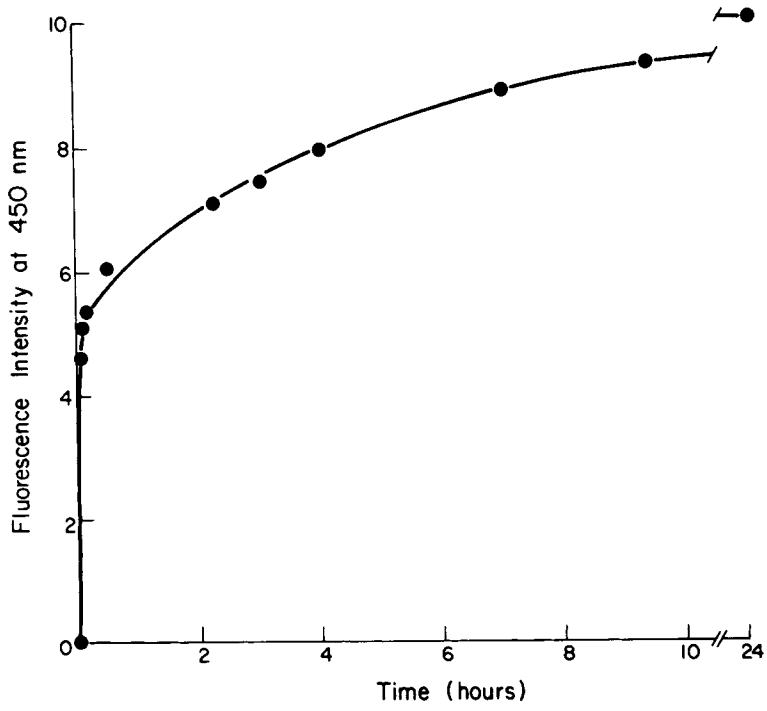


Fig. 1. Rate or reaction of 300 μM IAANS with 100 μM creatine kinase in 50 mM K TES, pH7, at 0°C. Aliquots were taken at the stated times, diluted 80-fold with 50 mM K TES, and the fluorescence at 450 nm was measured with excitation at 330 nm.

but significantly strengthens ATP binding while phosphocreatine strongly inhibits ATP binding and moderately inhibits ADP binding.

The binding of anions to nucleotide-free creatine kinase was discovered when it was observed that the fluorescence of IAANS creatine kinase was lower and the nucleotide binding much weaker in chloride buffers than in acetate buffers. It was subsequently found that not only did all small anions quench the IAANS fluorescence of modified creatine kinase but also the tryptophan fluorescence of native protein. Linear double reciprocal plots were obtained for the fluorescence quenching, and the anion dissociation constants determined for either native or IAANS-modified creatine kinase were in good agreement (Table III). The binding of anions was also shown by the noncompetitive quenching of bound 1,8-ANS fluorescence (7) which gave identical binding constants. Polyanions are bound more strongly than monoanions, but nitrate was found to be most effective in disrupting protein structure; however, the fluorescence quenching does not follow the chaotropic series.

As seen in Table II, 100 mM chloride increases the AMP dissociation constant 3-fold, the ADP constant 13-fold, and the ATP constant 21-fold, which would imply that the anion binding site is probably near the location of the transferable phosphoryl group of

TABLE I. Nucleotide Binding to IAANS Creatine Kinase

Nucleotide	K_d (μM)	Fluorescence change (%)
AMP	3.8	+ 213
ADP	12.7	+ 145
ATP	40.5	+ 145
3',5'-cyclic AMP	1,350	+ 80
Adenosine	90,000	+ 150
IMP	5.4	+ 111
IDP	16.4	+ 71
GDP	13.2	+ 177
XDP	17.1	+ 100
CDP	256	− 37
UDP	346	− 54

Conditions: 1 μM IAANS creatine kinase in 50 mM K TES, pH 7, at 10°C. Excitation was at 330 nm with emission monitored at 450 nm. The fluorescence change was calculated from the intercept of the double reciprocal plot.

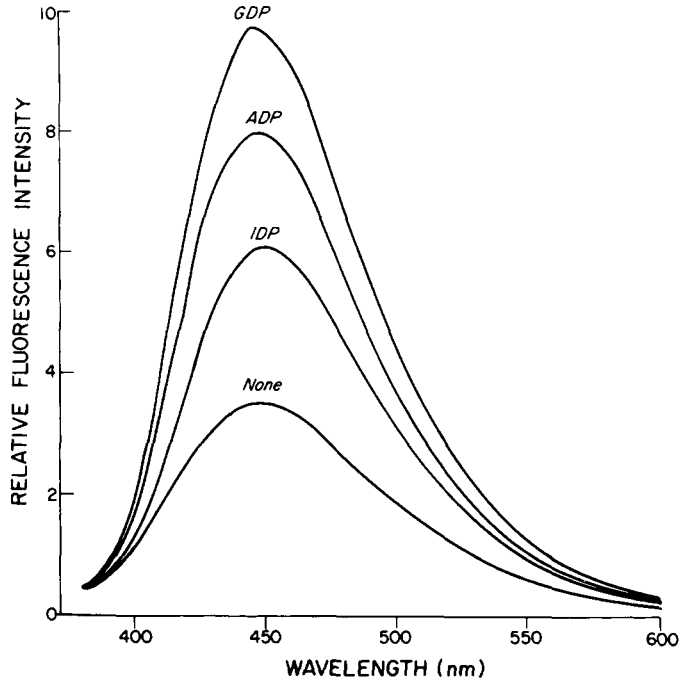


Fig. 2. Fluorescence enhancement of IAANS creatine kinase by purine nucleotides. Solutions of 1μM IAANS creatine kinase were made 2.5 mM with the indicated nucleotides. The fluorescence before nucleotide addition is in the spectrum labeled none. Excitation of all samples was at 330 nm.

TABLE II. Effect of Modifiers on Nucleotide Binding

Modifier	AMP, K_d (μM)	ADP, K_d (μM)	ATP, K_d (μM)
None	3.8	12.7	40.5
10 mM Mg acetate	3.8	16.9	427
50 mM creatine		15.5	19.0
5 mM phosphocreatine		32.0	231
33 mM phosphocreatine			746
100 mM KCl	12.8	164	862
100 mM $NaNO_3$		156	
100 mM Na_2SO_4		337	
150 mM K acetate		25.8	
150 mM NaCN		45.5	

TABLE III. Anion Binding to Creatine Kinase

	Creatine kinase		IAANS creatine kinase	
Salt	K_d (mM)	Q (%)	K_d (mM)	Q (%)
LiCl	53.4	9.4	55.5	38.2
KCl			59.7	35.7
NaBr	34.7	4.6	25.1	39.4
NaCN	195	21.5		
$NaNO_3$			74.2	74.1
K acetate	216	6.9	183	27.7
Na phosphate (pH 7)			22.5	27.2
Na pyrophosphate	6.8	7.1	2.5	30.8
Phosphocreatine (Na)			12.7	16.4
Na_2SO_4	8.4	10.4	10.0	46.0

Conditions: 1.25 μM creatine kinase or IAANS creatine kinase in 50 mM K TES, pH 7, at 10°C. The percent fluorescence quenching (Q) at infinite anion concentration was calculated from the intercept of the double reciprocal plot.

ATP. The binding of ADP and chloride was shown to be noncompetitive. It has been proposed that small planar anions, and especially nitrate, inhibit the creatine kinase activity by forming a stable dead-end complex with the anion located between the MgADP and creatine (8). In the case of IAANS creatine kinase, the increased stability and transition state character of this complex are questionable since it is clear in Fig. 3 that, under these conditions, nitrate is a simple noncompetitive inhibitor of ADP binding. It was also shown that repeating the experiment of McLaughlin (7) at pH 7, but correcting for the inhibition of 1,8-ANS binding by nitrate, also gave noncompetitive inhibition of MgADP binding by nitrate in the presence of creatine.

When the reaction of creatine kinase with IAANS is terminated after 1 min by addition of an excess of 2-mercaptoethanol, the result is creatine kinase specifically modified on one of the two subunits. This suggests using excited-state energy transfer as a "spectroscopic ruler" (9) to determine the distance between the active sites of creatine kinase. In a preliminary study, it has been found that IAANS transfers energy with about 25% efficiency to I as an acceptor on the thiol of the other subunit, and 28% to II (Fig. 4).

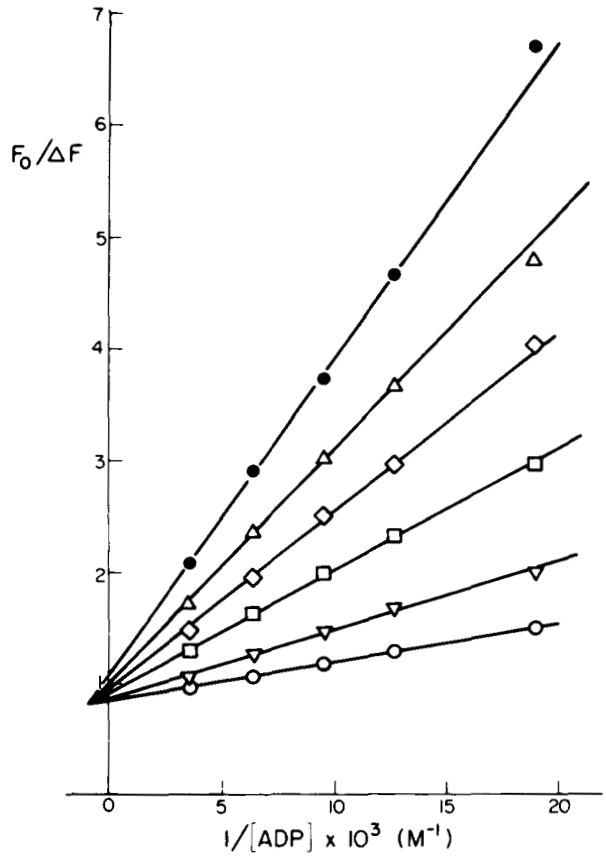
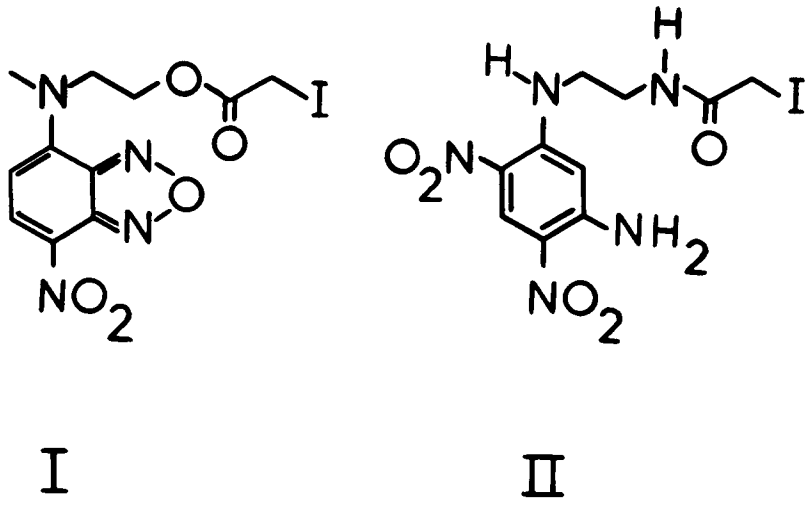


Fig. 3. Effect of $NaNO_3$ on the MgADP-induced fluorescence enhancement of IAANS creatine kinase in the presence of 50 mM creatine. The Mg acetate concentration in all titrations was 10 mM. The fixed concentrations of $NaNO_3$ used were: none (○); 25 mM (□); 50 mM (▽); 75 mM (●); 100 mM (△); and 150 mM (◇).

This low efficiency would indicate that the active sites are probably well separated (> 30 Å). Addition of ADP increases the quantum yield of the donor. This would normally be expected to increase the energy transfer efficiency (10). What was found, however, was that the efficiency of transfer from IAANS to I decreased to 17% and to II decreased to 16%. The probable cause is an increase in distance between the active sites related to the protein conformational change on nucleotide binding.

DISCUSSION

These results will be discussed more fully in subsequent papers, however the implications for creatine kinase structure and mechanism will be reviewed here. Creatine kinase appears to possess a conformational flexibility about a large hydrophobic crevice that contains the nucleotide and creatine binding sites and a large cavity that accommodates



I II

Fig. 4. Structure of the probes used as energy transfer acceptors.

the three aromatic rings of the IAANS chromophore. Purine nucleotides tighten the structure around the probe but differ in their ability to bring the enzyme into an active conformation with the optimum electronic and steric alignment for enzymatic reaction. The fluorescence enhancement is the same for K^+ADP, K^+ATP, MgADP, MgATP, and MgATP + creatine, which implies that the conformational change caused by each around the probe is identical. Mg^{2+} strongly inhibits ATP binding to the modified enzyme but has little effect on ADP binding. While this may be an artifact of the chemical modification, it suggests that the Mg^{2+} is chelated to the β and γ phosphates of ATP rather than to the α and β phosphates as suggested by Cohn et al. (5). Pyrimidine nucleotides and anions appear to competitively open the structure and to increase access of water to the active site. The binding of anions may disrupt a salt bridge that is necessary for maintaining the active enzyme structure.

The noncompetitive inhibition of MgADP binding by nitrate is not consistent with the stabilization of a dead-end complex with transition-state character, as suggested by Milner-White and Watts (8). At pH 7, the effect of nitrate appears to be simple noncompetitive inhibition of MgADP binding (Fig. 3) caused by both electrostatic repulsion between the charged nitrate and β phosphate and a direct destabilizing effect of nitrate on the protein conformation. The noncompetitive nature of the interaction implies that the MgADP, creatine, and nitrate can be bound simultaneously to the enzyme but the quaternary complex is not stabilized by nitrate binding and the apparent ADP dissociation constant is increased.

The biphasic rate of reaction of IAANS with rabbit skeletal muscle creatine kinase is the first clearly demonstrated difference in reactivity of the two subunits with an alkylating reagent, a phenomenon well documented for some brain-type creatine kinases (11, 12). It is difficult to distinguish an anticooperative effect of modification of the first subunit on the rate of reaction of the second and an intrinsically different rate of reaction of the two

subunits caused by solvation or steric factors.

The energy transfer results are preliminary. They tend to indicate, however, that the active sites of creatine kinase are well separated and become further apart during the conformational change caused by nucleotide binding.

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