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THE INTERACTION OF TETRAIODOFLUORESCEIN WITH CREATINE KINASE

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Summary

The dye 2′,4′,5′,7′-tetraiodofluorescein is a potent inhibitor of creatine kinase (ATP:creatine *N*-phosphotransferase, EC 2.7.3.2) with an apparent competitive inhibition constant with respect to $MgATP^{2-}$ of $2.6 \cdot 10^{-5}$ M. The association of the dye with the enzyme elicited a red shift in the dye's spectrum, indicative of a binding site less polar than water. The dye binds to the enzyme with an equilibrium constant of dissociation of $1.7 \cdot 10^{-5}$ M. MgATP or MgADP competes for the dye-binding site. Creatine binds to creatine kinase-tetraiodofluorescein complex to form a ternary complex and further causes a blue-shift in the spectrum of the bound dye. The binding of the dye to fully active creatine kinase causes conformational change that was monitored by enzyme-bound 2-mercuri-4-nitrophenol, a conformation-dependent "reporter group".

Introduction

A number of spectroscopic techniques have been utilized to study the binding of substrates to creatine kinase (ATP:creatine *N*-phosphotransferase, EC 2.7.3.2) in solution [1—4]. Very few of these, if any, have been able to assess the possible environment of the various substrate binding sites although a number have detected substrate-induced conformational changes. Recently, the dye 2′,4′,5′,7′-tetraiodofluorescein has been used to probe the nature of the nucleotide binding site of two different nucleotide-binding enzymes, namely lactate dehydrogenase [5] and aspartate transcarbamylase [6]. X-ray crystallographic study of lactate dehydrogenase has shown unambiguously that the dye binds at a site coincident with that of the adenosine portion of the cofactor NAD. The dye has also been shown to be a competitive inhibitor of the dehydrogenase. The tetraiodofluorescein activates the allosteric enzyme aspartate transcarbamylase, normally activated by the natural effector ATP [6]. A study of the interaction of the dye with creatine kinase, a member of another general

class of nucleotide-binding proteins, was undertaken under the assumption that tetraiodofluorescein would mimic the adenosine of substrates ATP and/or ADP. Moreover, because the dye is influenced by the environment of its binding site, the specific interaction of the dye with creatine kinase has proven to be useful in the appraisal of the polarity of the adenosine binding site. The enzyme-bound dye also served as a useful spectral probe to study the binding of various natural substrates and anion inhibitors. In addition, we have investigated the possibility that the dye induces a conformational change by investigating the binding of the dye to active creatine kinase labeled with 2-mercuri-4-nitrophenol [7], an environmentally sensitive chromophoric probe.

Mainly on the basis of the extreme similarity of the interaction of the dye with creatine kinase and with the other aforementioned classes of nucleotide-binding enzymes: lactate dehydrogenase and aspartate transcarbamylase, it can be concluded that the adenosine binding sites of these various types of enzymes are remarkably alike.

Materials and Methods

Materials. Sodium salts of ADP and ATP were obtained from Calbiochem; creatine from Fisher Scientific; and sodium salt of 2′,4′,5′,7′-tetraiodofluorescein from Eastman Chemicals. All other chemicals were of reagent grade and used without further purification.

Enzyme purification and assay. Creatine kinase was isolated from the breast muscle of normal, 5-month-old New Hampshire chickens (Line 200) by the procedure of Roy et al. [8]. Protein concentration was determined spectrophotometrically at 280 nm, with an extinction coefficient of 0.89 $(mg/ml)^{-1} \cdot cm^{-1}$ [9]. The molecular weight of 80 000 [10] was used to determine molar concentration.

The activity of the enzyme was assayed in the forward reaction at pH 9.00, 30°C by a pH-stat method [11—12]. Besides the substrates creatine and MgATP, the assay mixture contained 0.1 M sodium acetate and 1 mM cysteine [12]. Specific activity is defined as μmol H^+ released/min per mg of enzyme.

Spectrophotometric measurements. Measurements were performed at 30°C using a Cary 118 spectrophotometer and a matched quartz 2.5 ml volume cells of 1.0 cm light path. For each visible spectrum obtained, the pen was balanced at 650 nm, where all solutions were transparent, and a common baseline was recorded.

Results

Binding of tetraiodofluorescein to creatine kinase

The binding of tetraiodofluorescein to creatine kinase in solution elicited a pronounced red shift in the visible spectrum (Fig. 1). The maximum absorbance difference is at 543 nm and the minimum at 519 nm. This spectral shift was utilized to follow the titration of creatine kinase with the dye (Fig. 2). A dissociation constant of $1.7 \cdot 10^{-5}$ for the creatine kinase-tetraiodofluorescein complex was determined form a 'double-reciprocal' plot (Fig. 2, insert). The analysis of the titration data by Scatchard plot [13] showed a dissociation con-

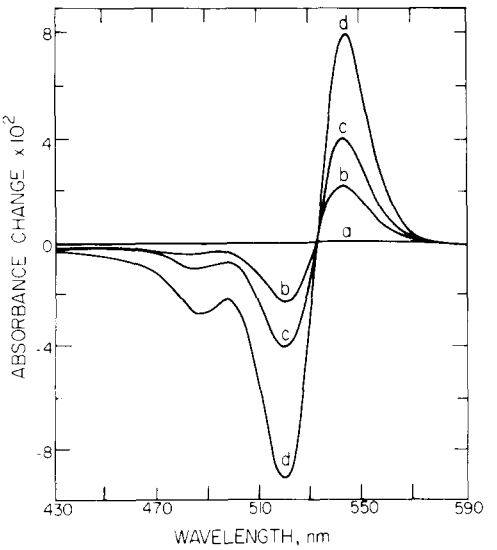

Fig. 1. Difference spectra of free versus tetraiodofluorescein bound to creatine kinase. a: Baseline scan of the sample cuvette which contained 0.6 mg enzyme in 2.5 ml 0.1 M *N*-2-hydroxyethylpiperazine propane sulfonic acid (EPPS) buffer pH 8.00 against the reference cell which contained 2.5 ml buffer. Curves b, c, d: spectra produced by the addition of small volume of 2 mM tetraiodofluorescein to both cells to give a concentration of 3.20, 6.38, 19.02 μM, respectively.

stant of $1.5 \cdot 10^{-5}$ M and indicated that the dye-binding sites in creatine kinase are equal and independent and that one mole of tetraiodofluorescein is bound per subunit of 40 000 daltons.

A Lineweaver-Burk plot of inhibition data indicates that the dye is a competitive inhibitor relative to $MgATP^{2-}$ (Fig. 3, I). An inhibition constant of $2.6 \cdot 10^{-5}$ M was obtained from the horizontal intercept of a replot of slopes versus dye concentration (Fig. 3, II). Since creatine kinase conforms to a rapid equilibrium, random mechanism, this value is an apparent inhibition constant [14].

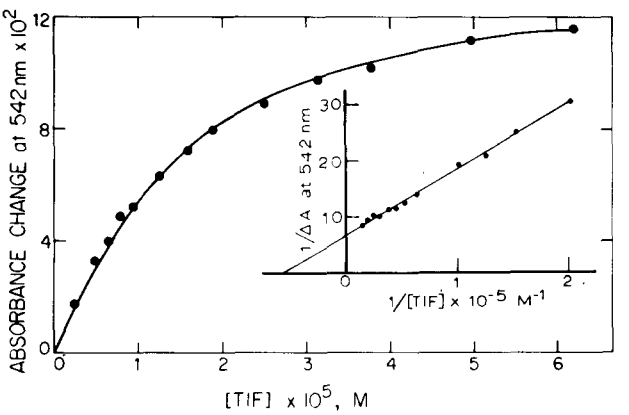

Fig. 2. Titration of creatine kinase with tetraiodofluorescein. Conditions as in Fig. 1. Insert: Double reciprocal plot of absorbance change at 542 nm versus tetraiodofluorescein concentration.

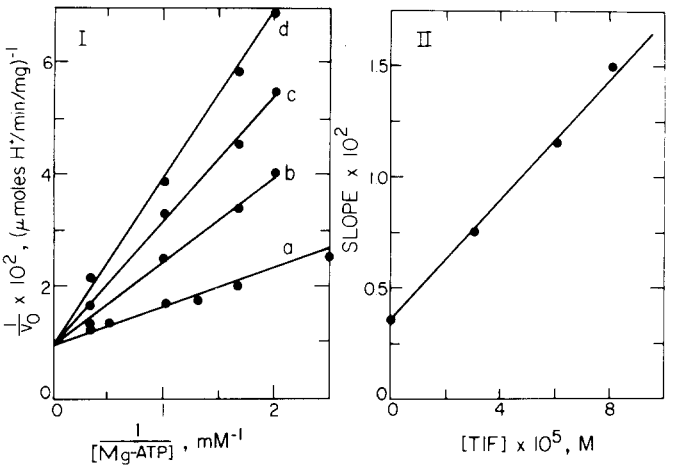

Fig. 3. Kinetics of inhibition of creatine kinase by tetraiodofluorescein. I: Lineweaver-Burk plots of initial rates of the forward reaction versus MgATP concentrations in the presence of 0, 30, 60 and 80 mM tetraiodofluorescein (curves a through d). The creatine concentration was held constant at 40 mM and the initial MgATP concentration was varied so that the free Mg^{2+} ion (as magnesium acetate) was always 1 mM. Other components of the assay mixture are 100 mM sodium acetate and 1 mM cysteine. II: Replot of the slopes obtained from the Lineweaver-Burk plots versus tetraiodofluorescein concentration.

Binding of substrates to creatine kinase-tetraiodofluorescein complex

The effect of ligands on the creatine kinase-dye complex was examined. Each ligand could either displace the bound dye, or form a ternary complex concomitant with perturbation of the bound dye spectrum. To test these two possibilties, difference spectrum measurements were conducted on creatine kinase: tetraiodofluorescein complex plus added ligand in the sample cuvette against creatine kinase:tetraiodofluorescein complex in the reference cell. If in the sample cell the tetraiodofluorescein were displaced by the ligand, the difference spectrum produced would be a mirror image of that shown in Fig. 1. It can be seen in Fig. 4, curve b, that MgADP causes the displacement of the bound tetraiodofluorescein. MgATP behaves similarly. On the other hand, the binding of creatine, a substrate or nitrate, an anion inhibitor [10,12] produced varied spectral changes which are not indicative of a dye displacement but of the formation of enzyme-tetraiodofluorescein:ligand ternary complex (Fig. 4).

Binding of tetraiodofluorescein to active creatine kinase labeled with 2-mercuri-4-nitrophenol

Creatine kinase can be labeled with 2 equivalents of 2-chloromercuri-4-nitrophenol, an environment-sensitive chromophoric probe, with the preservation of full activity [4,7]. Moreover, the enzyme-bound "reporter group" has been shown to be an effective and sensitive indicator of a variety of substrate-induced conformational changes. For instance, the binding of MgADP to the fully active creatine kinase labeled with the probe causes a bound nitrophenol spectral change that was best ascribed to a conformational change. The effect of tetraiodofluorescein on the bound 2-mercuri-4-nitrophenol was studied to test for a nitrophenol spectral change attributable to a dye-induced conformational rearrangement. From Fig. 5, it can be seen that the dye elicited a hyperchromic

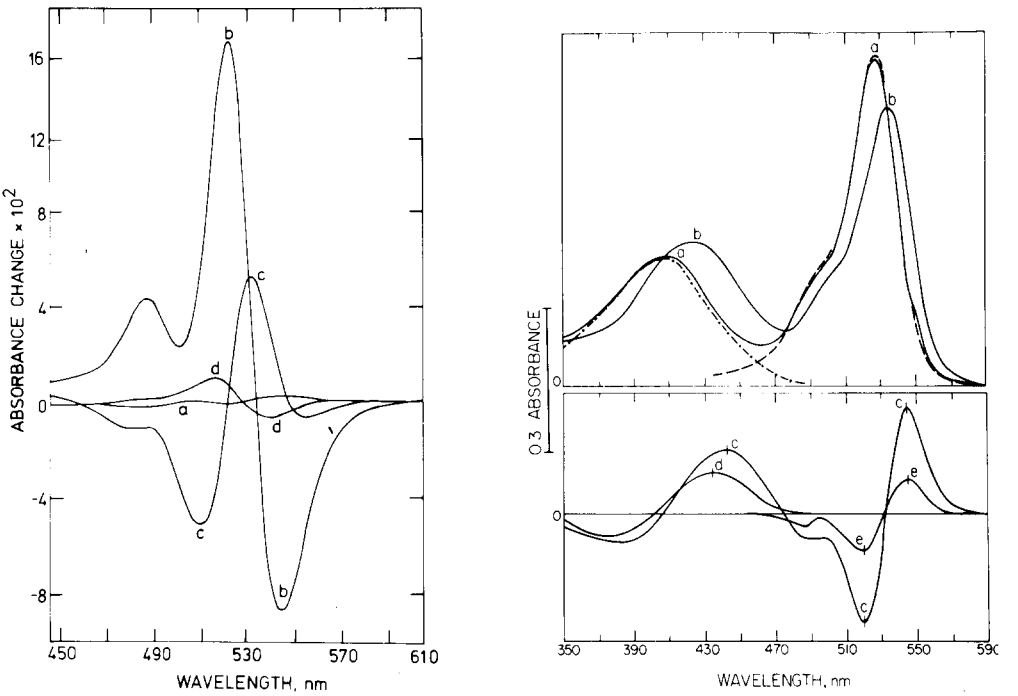

Fig. 4. Binding of ligands to creatine kinase-tetraiodofluorescein complex. Difference spectra produced by the addition of ligands to enzyme-tetraiodofluorescein complex. All solutions were buffered in 0.1 M EPPS pH 8.00 ± 0.005. A baseline scan, typically shown in curve a, was established with the reference and sample cells containing equal volume of tetraiodofluorescein-enzyme complex. Ligand or perturbant was added to the sample cell and buffer to reference cell to compensate for the increase in volume. Curve b: addition of MgADP to a final concentration of 13.4 mM (the inclusion of magnesium acetate, see legend to Fig. 3. Curve c: addition of creatine (33.6 mM final concentration). Curve d: addition of $NaNO_3$ (10.8 mM final concentration). For curves b to d the final concentration of tetraiodofluorescein and enzyme were 18.8 μM and 3.5 μM, respectively.

Fig. 5. Interaction of tetraiodofluorescein with active creatine kinase labeled with 2 equivalents of 2-mercuri-4-nitrophenol. I (top panel). Absolute spectra: curve a, 20 μM mercurinitrophenol and 11.2 μM tetraiodofluorescein; curve b, 11.2 μM tetraiodofluorescein and 10 μM creatine kinase labeled with 20 μM mercurinitrophenol. —·—, 20 μM mercurinitrophenol. ------, 11.2 μM tetraiodofluorescein. II (Bottom panel). Difference spectra: curve c, mercurinitrophenol-creatine kinase labeled with plus tetraiodofluorescein versus free mercurinitrophenol and tetraiodofluorescein. Difference spectrum of curve b against a, top panel. Curve d, 20 μM mercurinitrophenol bound to 10 μM creatine kinase against 20 μM mercurinitrophenol. Curve e, 11.2 μM tetraiodofluorescein and 10 μM creatine kinase against 11.2 μM tetraiodofluorescein. All solutions were buffered in 0.1 M EPPS pH 8.00 ± 0.005. Spectra were obtained using 1 cm, 2.5-ml volume cuvettes.

shift and an increase in extinction of the bound nitrophenol spectrum. Furthermore, the binding of the dye to the mercurinitrophenol-labeled creatine kinase caused a red shift and an enhancement of its visible spectrum relative to that observed in the binding of the dye to native, unlabeled enzyme.

Discussion

The red shift in the spectrum elicited by the binding of the tetraiodofluorescein to creatine kinase is very similar if not identical with those previously

observed with lactate dehydrogenase [5] and aspartate transcarbamylase [6]. The direction of the spectral shift, which is similarly observed when the free dye is in a solvent of low dielectric constant, has been attributed to a dye binding site less polar than water. The fact that, in these three different enzymes, the bound tetraiodofluorescein spectral changes and the dissociation constants of the various enzyme:tetraiodofluorescein complexes are nearly identical, strongly indicates that the tetraiodofluorescein binding site of these enzymes must be extremely similar. X-ray crystallographic analysis has unambiguously shown that the dye binds to lactate dehydrogenase at a site coincident with the adenosine moiety of the cofactor NAD [5]. Moreover, Rossmann and co-workers have recognized, on the basis of available structural data of a number of dehydrogenases and kinases, the existence among these enzymes of a similar adenosine binding site:the so-called "nucleotide fold" [15].

The finding that tetraiodofluorescein is a competitive inhibitor with respect to MgATP and that the difference spectrum produced by the binding of the dye can be completely abolished by MgATP or MgADP further indicates that the dye binds at the nucleotide binding site of creatine kinase. However, the dye appears to bind more tightly than MgATP since the equilibrium constant of dissociation of $3 \cdot 10^{-4}$ M for the enzyme-MgATP complex [16] is about 15 times greater than the dissociation constant of the enzyme-dye complex.

Creatine or nitrate, on the other hand, produced bound dye spectral changes which indicate that either ligand is capable of binding to the enzyme-tetraiodofluorescein complex to form a ternary complex. The formation of an enzyme-dye-creatine ternary complex is not unexpected since creatine appears to bind to a site different from the nucleotide binding site. Moreover, creatine caused a blue shift in the bound tetraiodofluorescein spectrum (Fig. 4, curve c), indicative of a change to a more polar dye environment. Nitrate, a potent inhibitor of creatine kinase, has been postulated to bind to a site coincident with the transferable γ-phosphoryl group of ATP [10,12]. The spectral perturbations of the bound dye spectrum accompanying the formation of ternary complexes can be attributed to changes in the environment of the dye in the active center brought about by substrate-induced conformational change or to direct interaction of the dye and substrate. A conformational change induced by creatine, in particular, would be in keeping with the observations [1—4,10] that creatine causes a structural rearrangement upon binding to enzyme-nucleotide complexes.

A pair of cysteine residues of creatine kinase can be labeled stoichiometrically with two equivalents of 2-chloromercuri-4-nitrophenol, one chromophore per subunit, without the loss of activity [7]. Furthermore, the binding of substrates and anions, singly or in various combinations, to the labeled enzyme elicited varied changes in the bound nitrophenol spectrum that were best ascribed to varied conformational changes in the local environment of the reporter group. The perturbation by tetraiodofluorescein of the nitrophenol bound to creatine kinase can, likewise, be attributed to a dye-induced conformational change. Moreover, the nitrophenol attached to the enzyme apparently has an influence on the bound tetraiodofluorescein spectrum, causing a red shift and an enhancement of the dye spectrum relative to the change observed in the binding of the dye to the native, unlabeled enzyme (Fig. 5).

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