Conformational changes at the active site of creatine kinase at low concentrations of guanidinium chloride

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It has been previously reported that, during denaturation of creatine kinase by guanidinium chloride (GdmCl) or urea [Tsou (1986), Trends Biochem. Sci. **11**, 427–429], inactivation occurs before noticeable conformational change can be detected, and it is suggested that the conformation at the active site is more easily perturbed and hence more flexible than the molecule as a whole. In this study, the thiol and amino groups at or near the active site of creatine kinase are labelled with *o*-phthaldehyde to form a fluorescent probe. Both the emission intensity and anisotropy decrease during denaturation indicating exposure of this probe and increased mobility of the active site. The above conformational changes take place together with enzyme inactivation at lower GdmCl concentrations than required to bring about intrinsic fluorescence changes of the enzyme. At the same GdmCl concentration, the rate of exposure of the probe is comparable with that of inactivation and is several orders of magnitude faster than that for the unfolding of the molecule as a whole.

INTRODUCTION

It has been reported in this (Tsou, 1986; Yao et al., 1982, 1984; Liu and Tsou, 1987; Liang et al., 1990; Ma and Tsou, 1991) and other laboratories (Ismond et al., 1988; West et al., 1990; Chen et al., 1990; Kelly and Price, 1991) that during the denaturation of a number of enzymes by guanidinium chloride (GdmCl) or urea, inactivation occurs before noticeable conformational change of the enzyme molecule as a whole can be detected. It is therefore suggested that the conformation of enzyme active sites is held together by relatively weak forces and is easily perturbed by denaturants. Furthermore, with the fluorescent NAD derivative introduced at the active site of D-glyceraldehyde-3-phosphate dehydrogenase, it has been demonstrated that conformational changes at the active site of this enzyme indeed take place, together with inactivation of the enzyme, before unfolding of the rest of the molecule (Xie and Tsou, 1987).

With a fluorescent probe formed from *o*-phthaldehyde (OPTA) and the thiol and amino groups at or near the active site of creatine kinase, it is shown in the present paper that inactivation and exposure of the probe take place simultaneously and well before unfolding of the molecule as a whole. Fluorescence emission anisotropy measurements suggest that the above is accompanied by increased mobility at the active site.

MATERIALS AND METHODS

The preparation of rabbit muscle creatine kinase was as described before (Yao et al., 1982, 1984). Preparation of OPTA-modified creatine kinase was carried out in 50 mM sodium borate buffer, pH 8.0, containing 7.3 μM enzyme and 30 mM OPTA for 1 h at 25 °C. The excess of OPTA was then removed by column chromatography on Sephadex G-25. Enzyme concentration was determined by absorbance at 280 nm with $A^{1\%}_{1\,cm} = 8.8$ (Noda et al., 1954). 5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB) and OPTA were Sigma products and ATP (99% pure) was from Boehringer Mannheim. All other reagents were local products of analytical grade used without further purification.

The activity of creatine kinase was routinely determined at 25 °C by following proton generation during the reaction of ATP and creatine with the indicator Thymol Blue at 597 nm. A calibration curve was constructed to correlate the absorption change with proton generation by titration with standardized HCl of an otherwise identical reaction mixture without the enzyme. The reaction mixture contained: ATP (4 mM), creatine, (24 mM), magnesium acetate (5 mM) and Thymol Blue (0.008 %) in 5 mM glycine buffer, pH 9.0.

1-(β-Hydroxyethylenethio)-2-*n*-propylisoindole was synthesized as described by Simons and Johnson (1978) and was found to have the same absorption and n.m.r. spectra as described by the above authors. When the fluorescence emission intensity was measured at 428 nm, the excitation spectrum showed a peak at 332 nm and a shoulder at 345 nm (Simons and Johnson, 1978; Simons et al., 1979).

The determination of thiol groups was carried out with DTNB as described by Ellmann (1959). Conditions for the determination of reactive and total thiol groups in the absence and presence of 3 M GdmCl respectively were as described before and no further increase in measurable thiol was observed at higher GdmCl concentrations (Yao et al., 1985). Difference spectra of the complexes of creatine kinase with substrates and with the transition-state analogues against the native enzyme were measured essentially as described by Watts (1973).

Absorption spectra were measured with a Kontron UV 860 spectrophotometer and fluorescence emission spectra and anisotropy measurements were made with a Hitachi 850 spectrofluorimeter. The fluorescence intensities (I) were measured with the polarizers parallel (∥) and perpendicular (⊥) to each other and the anisotropy (r) was calculated as:

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$$

Fast reactions were followed with an Applied Photophysics SF.17MV stopped-flow instrument under a constant temperature of 25 °C.

Abbreviations used: GdmCl, guanidinium chloride; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); OPTA, *o*-phthaldehyde.

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RESULTS

Inactivation of creatine kinase by modification with OPTA

During the titration of creatine kinase with different amounts of OPTA, enzyme activity decreased, with the increase in fluorescence emission at 410 nm indicating the formation of the fluorescent derivative as described by Palczewski et al. (1983). This leads to inactivation as shown in Figure 1. The initial portion of the curve is linear and no further increase in emission intensity occurred after complete inactivation in spite of the presence of excess OPTA.

Absorption and fluorescence spectra of OPTA-modified enzyme

Figure 2 compares the absorption spectra of native and OPTA-modified creatine kinase. The modified enzyme shows a new band around 338 nm which is close to that for the model compound 1-(β-hydroxyethylenethio)-2-*n*-propylisoindole given by Simons and Johnson (1978) in ethanol and is undoubtedly

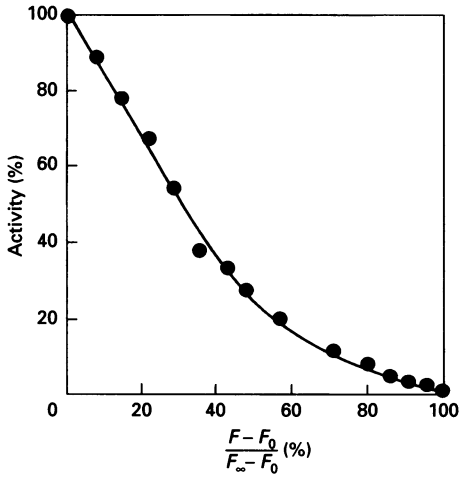


Figure 1 Inactivation of creatine kinase by OPTA

Creatine kinase, in 50 mM borate buffer, pH 8.0, was mixed with 25 μM OPTA in the same buffer to a final concentration of 0.9 μM. Samples were taken at different time intervals for measurement of activity remaining and increase in fluorescence emission intensity at 420 nm with an excitation wavelength of 338 nm.

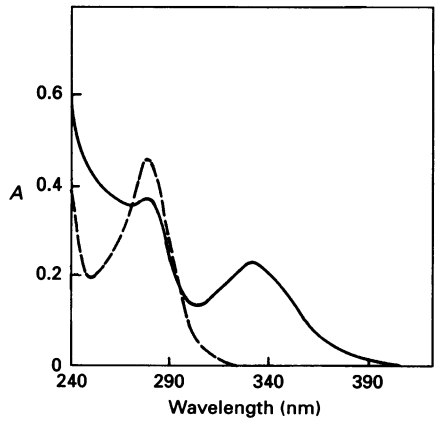


Figure 2 Absorption spectra of OPTA-modified creatine kinase

Concentrations of native (– – –) and modified (——) enzymes were 6.0 and 4.8 μM respectively, in 50 mM borate buffer, pH 8.0.

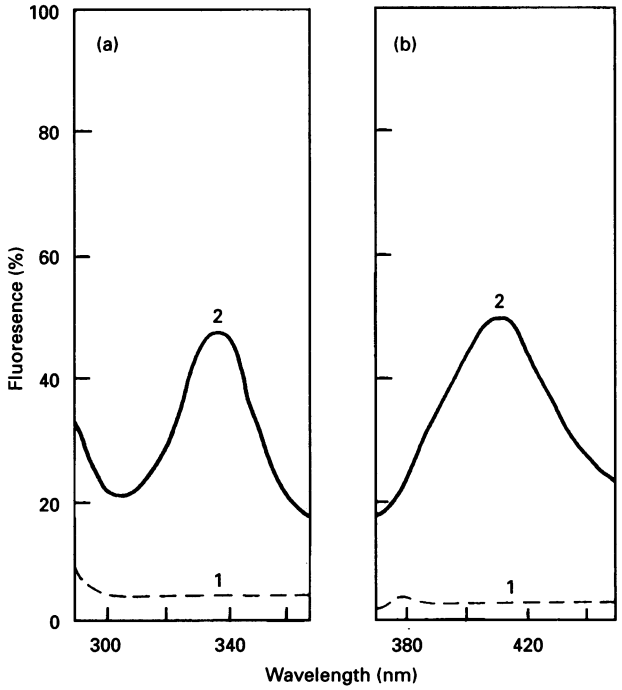


Figure 3 Fluorescence spectra of OPTA-modified creatine kinase

Fluorescence spectra of the modified enzyme (——) were recorded at an enzyme concentration of 0.5 μM borate buffer, pH 8.0, at 25 °C and compared with those of the native enzyme (– – –). (**a**) Excitation spectra with emission wavelength at 410 nm. (**b**) Emission spectra with excitation wavelength at 338 nm.

contributed by the fluorescence probe. The excitation and emission spectra of the modified enzyme have maxima at 338 and 410 nm respectively as shown in Figure 3(a) and 3(b). The model compound has a two-peaked excitation maximum at 332 and 345 nm and an emission maximum at 428 nm in 95% ethanol (Simons and Johnson, 1978). The difference could be due to different ring substituents and/or different environment for the fluorophore in the enzyme molecule and the model compound in ethanol solution. The emission and excitation spectra of the modified enzyme are similar to those reported for OPTA-modified aldolase (Palczewski et al., 1983).

Modification at the active site by OPTA

It is known that the dimeric molecule of creatine kinase has eight thiol groups, two of which are more reactive than the others and their modification usually leads to inactivation of the enzyme (Watts, 1973; Zhou and Tsou, 1987). This was shown to be the case for the OPTA-modified enzyme (Table 1). OPTA modification leads to complete inactivation with a decrease in the two reactive thiol groups, which presumably are the thiol groups at or near the active site. The total thiol groups available for reaction with DTNB under denaturation conditions also decreases from eight to six, indicating that the other thiol groups are not affected. In addition, an amino group is also known to be essential for the activity of creatine kinase (Kassab et al., 1968).

Watts (1973) showed that, in the presence of the substrates creatine–ADP–Mg^{2+} or the transition-state analogue creatine–ADP–Mg^{2+}–NO_3^-, the enzyme undergoes a conformational change, as indicated by absorbance changes in the u.v. region. Figure 4 compares the difference spectra for the native and the OPTA-modified enzyme in the presence of either the substrates or the transition-state analogue, and it is clear that no difference

Table 1 Thiol groups before and after OPTA modification

The reactive and total thiol groups were determined with DTNB in glycine buffer, pH 9.0, in the absence and presence of 3 M GdmCl respectively.

	Number of thiols	
	Reactive	Total
Native enzyme	1.72	7.2
OPTA-modified enzyme	0.12	5.4

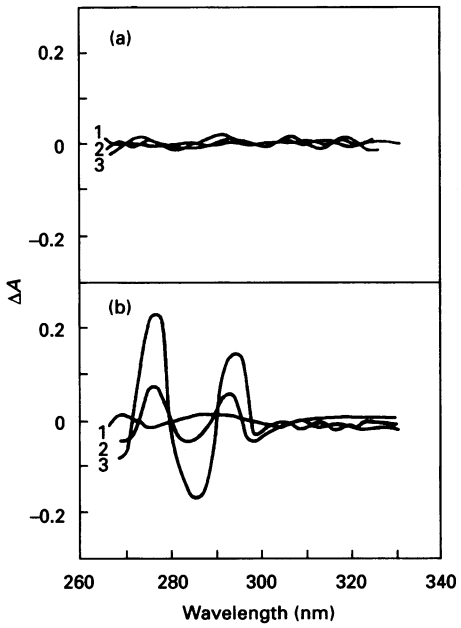


Figure 4 Difference spectra of creatine kinase with the substrates and the transition-state analogue

Final concentrations were 20 μM enzyme in 84 mM Tris/HCl buffer, pH 8.0, 59 mM creatine, 0.18 mM ADP, 3.6 mM magnesium acetate. For the measurement of the difference spectrum of the complex with the transition-state analogue, in addition to the substrates, 28 μM $NaNO_3$ was also added. (**a**) OPTA-modified enzyme; (**b**) native enzyme. In both (**a**) and (**b**), the curves are: 1, the base line; 2, the difference spectrum for the enzyme–substrate complex; 3, the difference spectrum for the complex with the transition-state analogue.

spectra can be observed for the modified enzyme, suggesting that the modification is at the active site and the introduction of the probe prevents the binding of the substrates.

Conformational changes at the active site

The effect of GdmCl on the fluorescence emission spectrum of the OPTA-labelled active site is shown in Figure 5. At 338 nm excitation, the 410 nm emission decreases with increasing GdmCl concentration, suggesting the exposure of the fluorophore to a more hydrophilic environment. Separate experiments on the model compound have shown that there is no direct quenching effect of GdmCl on the fluorophore (not shown). Moreover, the decrease in 410 nm emission is already significant at 0.3 M and approaches completion at 0.5 M GdmCl (see below), in the same range as required for the inactivation of the enzyme. In contrast, the intrinsic fluorescence due to exposure of the tryptophan residues shows no appreciable change at 0.3 M, begins to show some change at 0.5 M and reaches completion only at 3 M GdmCl (Yao et al., 1982).

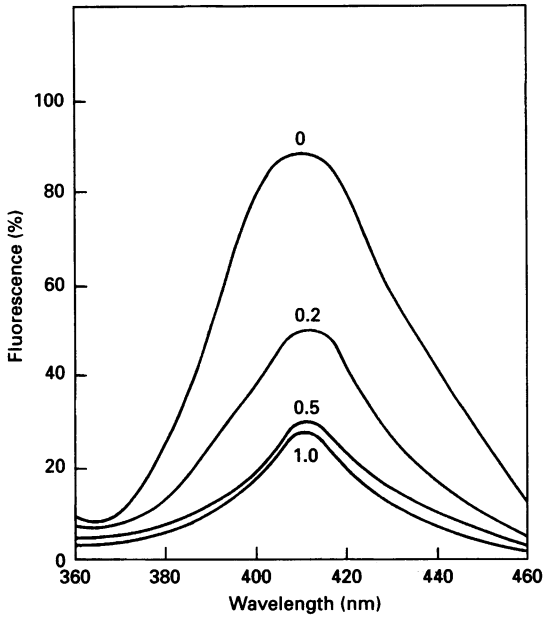


Figure 5 Effect of GdmCl on fluorescence emission of OPTA-modified creatine kinase

Fluorescence emission spectra at different concentrations of GdmCl were recorded at 1.0 μM protein in 50 mM borate buffer, pH 8.0, at 25 °C at an excitation wavelength of 338 nm. The numbers on the spectra are GdmCl concentrations (M).

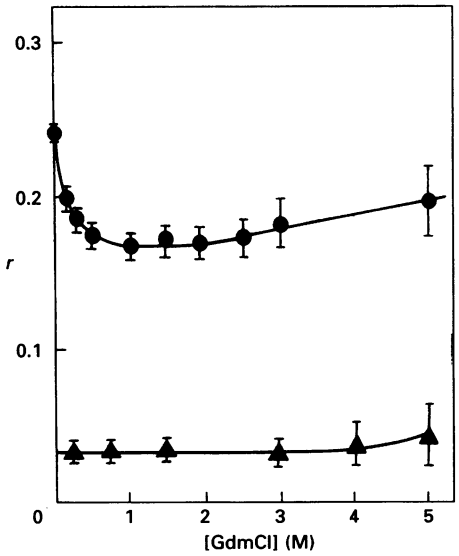


Figure 6 Effect of GdmCl on the emission anisotropy of the OPTA probe (●) and the model compound (▲)

Experimental conditions were, enzyme concentration, 7.0 μM, in 50 mM borate buffer, pH 8.0, at 25 °C. Emission was measured at 410 nm with an excitation wavelength of 338 nm. For measurements of emission anisotropy, see the text.

Increased mobility of the active site in GdmCl

Fluorescence polarization measurements show that the degree of emission anisotropy of the probe at 410 nm is considerably higher than that of the model compound and decreases with increasing concentrations of GdmCl, suggesting increased mobility of the fluorophore at the active site. This decrease occurs at a concentration range of 0–0.5 M GdmCl, and there is no further change in emission anisotropy when the GdmCl con-

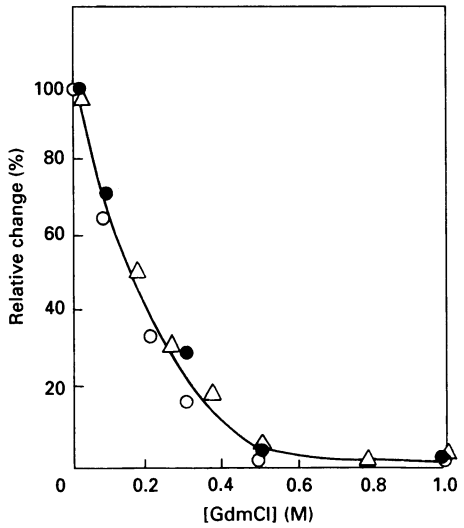


Figure 7 Comparison of exposure and emission anisotropy change of the OPTA probe with inactivation during unfolding by GdmCl

Effects of increasing concentrations of GdmCl on the fluorescence emission intensity at 410 nm (○) and emission anisotropy (△) of the OPTA probe and the extents of inactivation (●) at different concentrations of GdmCl are shown. For comparison, all data are expressed in percentage changes. Experimental conditions were as for Figure 6.

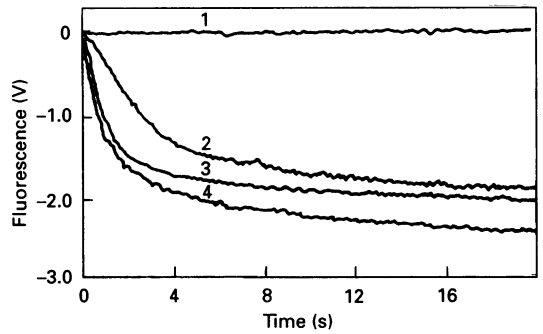


Figure 8 Kinetics of conformational changes at the active site during denaturation by GdmCl

The fluorescence emission changes in OPTA-modified creatine kinase (1.0 μM) in 50 mM borate buffer, pH 8.0, were followed at 410 nm in a stopped-flow apparatus. Curves 1–4 represent spectra obtained at final concentrations of GdmCl of 0, 0.3, 0.5 and 1.0 M respectively in the same buffer.

Table 2 Rate constants for the exposure of the active-site fluorescence label

All rate constants are given in s^{-1}. a, no significant change; b, not determined; c, reaction monophasic.

GdmCl (M)	Fluorescence emission change				
	OPTA probe			Inactivation	
	Fast phase	Slow phase	Intrinsic	Fast phase	Slow phase
0.3	0.38	0.049	a	b	0.0015*
0.5	1.18	0.11	0.0038*	3.6*	0.003*
1.0	2.9	c	0.04*	4.3*	c

* From Yao et al. (1982).

centration is further increased (Figure 6). Figure 6 also shows that the fluorescence of the model compound shows very little emission anisotropy and it is not affected by GdmCl. The slight increase in emission anisotropy at high GdmCl concentrations for both the probe and the model compound is undoubtedly due to an increase in solution viscosity.

Figure 7 compares enzyme inactivation, decrease in 410 nm emission and changes in emission anisotropy in solutions of different concentrations of GdmCl and shows that, at the same GdmCl concentration, these changes occur to the same extents. It is therefore clear that exposure of the OPTA fluorophore and the increase in mobility at the active site are parallel events to the loss of enzyme activity.

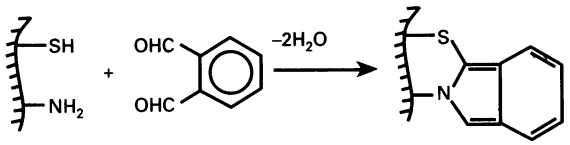
Kinetics of conformational change at the active site

In previous papers, it was reported that the rates of inactivation of creatine kinase during GdmCl or urea denaturation are many orders of magnitude faster than the corresponding rates of conformational change in the molecule as a whole (Yao et al., 1982, 1984). It is therefore of interest to examine the rate of fluorescence emission change at the active site with OPTA as a reporter. Like the inactivation reaction, the exposure of the OPTA fluorophore at the active site in the GdmCl concentration range 0.3–0.5 M, as followed by emission-intensity decrease at 410 nm, is a biphasic reaction. Again like the inactivation reaction, the change in the probe at 1 M GdmCl is monophasic. Figure 8 shows the initial fast phase of the reaction in a stopped-flow apparatus. The rate constants obtained by semilogarithmic plots are summarized in Table 2 and compared with the rates of inactivation and the overall conformational change of the enzyme molecule reported by Yao et al. (1982). Although the rates for inactivation and for the exposure of the active-site fluorophore are not exactly identical, it is clear that, at all the GdmCl concentrations examined, they are of the same order of magnitude and much faster than that for the overall conformational change of the molecule as followed by u.v. absorbance, c.d., intrinsic fluorescence and exposure of internal thiol groups reported previously (Yao et al., 1982).

DISCUSSION

Introduction of the probe at the active site

The reaction of OPTA with thiol-containing proteins has been shown by Palczewski et al. (1983) to be as follows:



The stoichiometry of the modification reaction with the reactive thiol group leading to complete inactivation and the fact that the modified enzyme no longer binds either the substrates or the transition-state analogue complex indicate that the probe is introduced at the active site.

Conformational change at the active site parallels that of enzyme inactivation by GdmCl

Results from this (Yao et al., 1982, 1984; Liu and Tsou, 1987; Liang et al., 1990; Ma and Tsou, 1991) and other laboratories (Ismond et al., 1988; West et al., 1990; Chen et al., 1990; Kelly and Price, 1991) have shown that inactivation of several enzymes

precedes conformational changes during denaturation by GdmCl, suggesting that enzyme active sites are located in limited regions more susceptible to perturbation by denaturants than the molecule as a whole (Tsou, 1986). Although the possibility that the decrease in activity is due to a reversible inhibition has been considered unlikely (Tsou, 1986), it has been suggested that for some enzymes the decrease in enzyme activity in GdmCl could actually be a reversible inhibition by the denaturant rather than partial unfolding of the molecule at the active site (Creighton, 1990). It is now shown in this paper that, with a fluorescent probe introduced at the active site of creatine kinase, inactivation takes place at the same time as active-site conformational changes, as shown by emission and anisotropy changes of the probe fluorophore.

In complete accord with the suggestion that the conformation of the enzyme active site is held together by weaker forces than the overall structure and hence is more easily perturbed during denaturation so that inactivation occurs before unfolding of the molecule as a whole, recent studies in this laboratory on the thermal denaturation of D-glyceraldehyde-3-phosphate dehydrogenase (Lin et al., 1990) show that inactivation in these circumstances also occurs before noticeable conformational change in the enzyme molecule as a whole.

Biphasic inactivation of creatine kinase

The kinetics of inactivation of creatine kinase (Yao et al., 1982) and some other enzymes (Liu and Tsou, 1987; Liang et al., 1990) have been found to be biphasic. The great difference in the rates of the two phases and the increase in the relative amplitudes of the fast phase of inactivation with the increase in GdmCl concentration (not shown) indicate that the two phases of the inactivation reaction are not due to inherent or induced asymmetry of the two subunits of creatine kinase. It has been previously suggested for D-glyceraldehyde-3-phosphate dehydrogenase (Liang et al., 1990) that the biphasic reaction could be a rapid reversible inactivation followed by a slow irreversible step. Both steps could involve some conformational changes at the active site. It should be noted that the slow phase of the fluorescence change of the probe is still faster than both the slow phase of inactivation and the conformational change of the molecule as a whole. Whether this biphasic reaction could be ascribed to a difference in the two subunits requires further investigation.

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Mobility at the active site

Fluorescence emission anisotropy is a result of restriction of the motion of the fluorophore (Lakowicz, 1983). The fluorescence anisotropy of the probe introduced at the active site of creatine kinase is much greater than the model compound in free solution under otherwise identical conditions, indicating decreased rotational rate or restricted motion of the probe in the enzyme molecule. In dilute GdmCl solutions, the anisotropy of the probe decreases markedly showing increased mobility of the probe molecule at the same time as inactivation of the enzyme. The above results provide direct evidence of conformational change during GdmCl denaturation and the flexibility of the active site of creatine kinase.

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