Conformational heterogeneity of creatine kinase determined from phase resolved fluorometry

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ABSTRACT Fluorescence lifetimes of dimeric rabbit muscle creatine kinase specifically dansylated at both active sites and the homologous monomeric lobster muscle arginine kinase singly dansylated were determined using phase-modulation methods with global analysis of overdetermined data sets. For both proteins, the data is adequately described by three discrete exponential decays or a Lorentzian double distributed decay. Analogue phase resolved spectroscopy also reveals the presence of at least two distinct fluorophore domains for the dansyl moieties of creatine kinase. The model fluorophore, dansyllysine, exhibits a monoexponential decay with a value that is highly solvent dependent. Because the monomeric arginine kinase exhibits essentially the same decay law as doubly derivatized dimeric creatine kinase, it is proposed that the multiple lifetimes of creatine kinase reflect two or more isomeric dimeric states and not subunit asymmetry within a conformationally homogeneous dimeric population. Exposure of arginine kinase to 6 M guanidinium chloride results in a shift to shorter lifetimes and narrowing of the lifetime distributions. Creatine kinase displays a small narrowing of the distribution, but little change in fractional populations or lifetimes. These results suggest the presence of structural elements resistant to denaturation.

The longest lifetime component in the triexponential discrete decay law of doubly dansylated creatine kinase is totally unquenched by acrylamide, whereas the two shorter lifetime components exhibit limited dynamic quenching. Steady-state quenching by acrylamide is significant and reveals a sharp distinction between accessible and nonaccessible dansyl groups. The major mechanism for interaction between the dansyl moieties and acrylamide is, atypically, static quenching. The results are consistent with two dansyl domains, one accessible and hydrophilic according to lifetime values and the other inaccessible and hydrophobic in solvent characteristics.

Energy transfer between the dansyl group and the eight tryptophan residues of dimeric creatine kinase give similar results (~35%) from measurements of lifetimes, steady-state donor quenching and sensitized acceptor emission. The similarity suggests that the overall flexibility of the dimeric protein is limited. The occurrence of multiple conformers of muscle creatine kinase provides an explanation for several previous observations, most notably the structural origins for compartmentation of the muscle isozyme observed in the myofibril.

INTRODUCTION

Creatine kinase from muscle is a homodimeric enzyme involved in energy transduction during myofibrillar contraction (1). Studies involving the labeling of the highly reactive, but catalytically nonessential thiol on each subunit, have led to the suggestion that the subunits are nonequivalent (2) and that the dimeric quaternary arrangement of subunits may be asymmetric (3). On the other hand, some of the observations attributed to subunit nonidentity could be explained by the occurrence of static or slowly isomerizing conformational homodimers, which in turn would account for several additional features of muscle CK[1] including cooperativity in substrate binding (1) and intracellular compartmentation (4).

After the highly reactive cysteines are reversibly protected, CK reacts with dansyl chloride specifically at the two essential lysines, one located at the active site on each subunit (5). This preparation has been used to study the phosphotransferase mechanism (6) and the subunit folding/association pathway (7). In preliminary studies, it has been found that doubly-dansylated CK exhibits two lifetime distributions or three discrete lifetimes. Because it was also observed that the model fluorophore, dansyllysine, displays a single, solvent sensitive lifetime, it seems possible that the multiple decay observed with doubly dansylated CK is reflecting the asymmetry in subunit arrangement. On the other hand, it is well known that proteins containing a single tryptophan exhibit bi- and triexponential decays (8). The origin of this behavior is under current scrutiny (9).

To determine if the multiexponential decay of double dansylated CK is related to the dimeric state, comparative studies with the homologous but singly labeled

[1]*Abbreviations used in this paper:* AK: arginine kinase; CK: creatine kinase.

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monomeric arginine kinase were performed. Decay laws for the denatured proteins were also determined. Additional efforts to characterize the multiple decays of dansylated CK included evaluation of analogue phase resolved spectra and quenching of dansylated CK with acrylamide. Lastly, to obtain a measure of possible protein flexibility, we determined the resonance energy transfer efficiency between the dansyl moiety and the intrinsic fluorophores using steady-state and time resolved methods.

The results provide evidence for multiple conformers and rather limited flexibility of CK conformation which is consistent with the role of this isozyme as a structural and functional component of the myofibril M-line (4).

MATERIALS AND METHODS

Sample preparation

Creatine kinase from rabbit muscle was purified as described (10), adding a final fractionation by hydroxyapatite (11). Assay of activity and protein concentration were measured as described (12). Lobster muscle arginine kinase, purchased from Sigma Chemical Co. (St. Louis, MO) exhibited a single protein staining band at molecular weight 40,000 daltons after electrophoresis in sodium dodecyl sulfate (13). Before reaction with dansyl chloride, samples of AK in ammonium sulfate were dialyzed against reaction buffer.

Reaction of CK and AK with dansyl chloride (Molecular Probes, Inc., Eugene, Oregon) was carried out as described (5, 7). Protein was first reacted with a 100-fold molar excess of sodium tetrathionate (Sigma Chemical Co.) in potassium phosphate or Hepes buffer, 0.05 M, pH 8.0. After 10 min, dansyl chloride (20-fold molar excess) in dimethylformamide (< 1/20, vol/vol) or Celite adsorbed dansyl chloride (10% wt/wt, from Molecular Probes Inc.) was added. Samples were reacted in the dark with slow rotation at 4° for various time intervals, centrifuged and filtered through Sephadex G-25. The high molecular weight, fluorescent product was dialyzed against the reaction buffer containing 2 mM dithiothreitol with two solution changes at a 400-fold greater volume than the protein solution. The reservoir dialysis solution was monitored for dansyl fluorescence. It was found that the Celite adsorbed dye provided higher protein recovery in the supernatant after centrifugation and better overall yield, but reaction times were considerably more protracted (~ 30 h for double dansylation) than when the solubilized dye was used. Labeling stoichiometry was determined from absorbance spectra using A (1%, 1 cm) = 8.8 and A (1%, 1 cm) = 8.1 at 280 nm, and ϵ = 4,300 $M^{-1}cm^{-1}$ at 340 nm for CK, AK and the dansyl moiety, respectively (5).

Fluorescence lifetime measurements

Fluorescence lifetimes were determined with a model 48000s phase/lifetime spectrofluorometer (SLM Instruments, Inc., Urbana, IL) and two model 250 frequency synthesizers (Program Test Sources, Littleton, MA). The excitation source was a model 4230NB HeCd laser (Liconix, Sunnyvale, CA) containing a 325-nm excitation wavelength. A 450 W xenon arc lamp and monochromator at 295 nm were used for excitation of the intrinsic fluorescence. The cuvette chamber was maintained at 25° with thermostated circulating water and purged continuously with an atmosphere of dry nitrogen. Turret rotation and frequency increases were accomplished using automated, macro program controlled inputs. The reference cuvette contained an ethanolic solution of PPO (lifetime of 1.32 ns), insulated to limit evaporation during data acquisition. Dansyl emission was collected through cutoff and bandpass filters over a range of 408–550 nm. The samples were scrupulously freed of particulates and stirred continuously during data acquisition. Typical parameters included three acquisitions each for standard and sample for at least 20 s each at 15 frequencies over the range of 2–100 MHz or until a phase shift of 70° was achieved. Complete data sets were obtained with at least three different emission cutoff or band pass filters.

Data analysis was performed using the Globals Unlimited software developed by Beechem and co-workers (14). Data sets were first converted from SLM to an ASCII format, then input to a 286 microprocessor microcomputer. Data sets were linked with respect to lifetimes and distributions but the intensity function or populations determined uniquely at each emission parameter. All linked data sets were subjected to rigorous confidence testing including the range of values within two standard deviations of the chi square. In the fitting routines, we use error value of 0.5° and 0.005, for the phase shift and demodulation, respectively.

Analogue phase resolved spectra (15) were obtained using the calculated phase angles required to suppress the long and short lifetime components. The procedure was similar to the steady-state acquisition except the laser excitation was frequency modulated (10 MHz) and the photomultiplier tubes maintained in the dynamic mode. Emission was detected through a monochromator with 4 and 2 nm slits. Energy transfer between the tryptophans and the dansyl moiety was measured using donor intensity and donor lifetime quenching and sensitized acceptor emission. Transfer efficiency (E) between donor (D) and acceptor (A) in the donor/acceptor pair (DA) is defined by:

$$E = (1 - F_{DA}/F_D) \quad (1)$$

$$E = (1 - \tau_{DA}/\tau_D) \quad (2)$$

$$E = \mathrm{Abs}_A^{\lambda D}/\mathrm{Abs}_D^{\lambda D}[(F_{DA}^{\lambda D}/F_A^{\lambda D}) - 1], \quad (3)$$

for the three procedures, respectively (16). In the lifetime measurements, a weighted average donor lifetime value was used. Emission was selected with a UG5 bandpass filter (Schott America Glass & Scientific Products, Inc., Yonkers, NY). Suitable decay laws in the absence and presence of the dansyl group were found to contain discrete biexponential components. Steady-state measurements are both excitation and emission corrected.

RESULTS

Absorbance and fluorescence spectra

The absorption, fluorescence excitation and emission spectra are illustrated in Fig. 1. The absorbances at 280 and 340 nm indicate that two moles of the dansyl group have reacted per mole of CK. Similar spectral analysis of dansylated AK reveals a single mole of dansyl moiety per mole of AK. The fluorescence emission spectral maximum of doubly dansylated CK shows a dependence upon excitation wavelength, suggesting heterogeneity in the emission (Fig. 2). Red shifting of the excitation wavelength is accompanied by a red shift in the emission maximum.

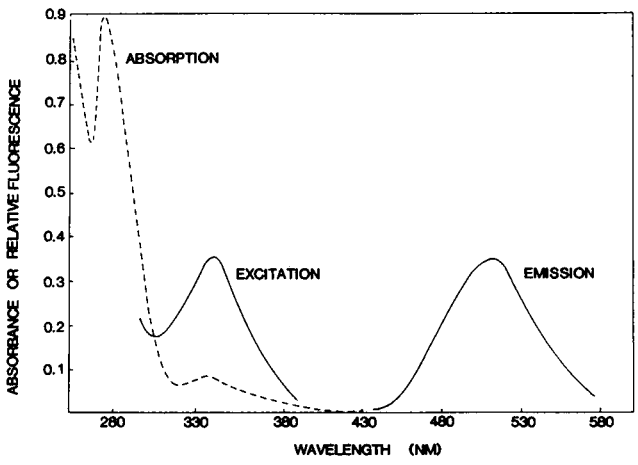

FIGURE 1 Absorbance and fluorescence excitation and emission spectra of doubly dansylated CK. Absorption spectrum was obtained from a sample containing 1.0 mg/mL, 12.6 μM, in 0.05 M Hepes buffer, pH 8.0 with 2 mM dithiothreitol. The fluorescence spectra were obtained on the same sample diluted 1 in 20 with the same buffer. Spectra are excitation and emission corrected and corrected for buffer components. Excitation: 340 nm; emission: 510 nm.

Fluorescence lifetimes

The fluorescence lifetime of the model fluorophore dansyllysine is solvent dependent. Increasing concentrations of buffered propanol from 0 to 90% result in a rather linear increase in lifetime of dansyllysine from 3.5 to 14 ns, respectively. The phase/modulation data was best described by a single discrete lifetime. Model testing with biexponential decays never exhibited a second lifetime of >0.2%. Under many conditions,

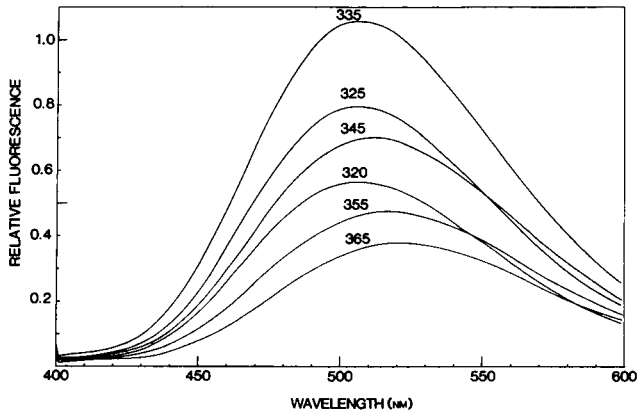

FIGURE 2 Fluorescence emission spectrum as a function of excitation wavelength. Sample and conditions are given in Fig. 1. Labels above each spectrum indicate fixed excitation wavelength in nanometers.

including dissolution in several different buffers (e.g., Hepes, pH 7.5), other buffered organic solvents (e.g., *n*-butanol), a single exponential fluorescence decay was always derived.

Lifetime of doubly dansylated CK

Results displayed in Table 1 show that the fluorescence decay of doubly dansylated CK is adequately described by a three-term discrete exponential decay law or a bimodal distribution. Although the data sets are overdetermined five times, there remains insufficient data to produce a clear assignment of the model for the decay law. Subsequent experiments with AK and denatured proteins also displayed similar chi-square values for triple exponential decays and bimodal distributions. The results for CK clearly show that the single discrete exponential decay law and the unimodal Lorentzian model are not appropriate.

Analogue phase resolved spectroscopy was used to separate the components of the steady-state spectrum of doubly dansylated CK. The value for longest lifetime (14.47 ns) obtained in the discrete lifetimes determination was used as the basis for calculation of the suppression phase angle (15). The results in Fig. 3 show that a 46° suppression produces a spectrum with an inverted emission maximum at 560 nm, whereas suppression (70°) of the weighted average of the short lifetimes produces an emission peak at 510 nm. Although it appears that the two components can be resolved, the bipolar nature of the spectrum with the long component suppressed indicates additional heterogeneity.

Comparison with singly labeled AK

The central lifetimes in the bimodal distribution model are longer for AK than CK (Table 1). This is also observed for the discrete models where the longest lifetime of AK is usually 16–17 ns whereas the corresponding lifetime for CK is 14–15 ns. In the bimodal Lorentzian model, the decay associated populations are not remarkably different between the two kinases although the long lifetime of AK is more distributed. The significant feature is that although AK is singly labeled, it clearly exhibits more than one lifetime, each with a substantial amplitude.

Lifetimes of denatured AK and CK

After exposure of dansylated AK to 6 M GDN/HCl, the lifetimes become substantially shorter, with a shift in the lifetime associated population towards the shorter lifetime and a small narrowing of the distributions (Table

TABLE 1 **Lifetimes (τ), associated population fractions (A)* and distributions (w) for dansylated phosphagen kinases**

Sample model	τ_1	$(A)_1$	$[w]_1$	τ_2	$(A)_2$	$[w]_2$	τ_3	χ^2
Dansylated CK								
$D^{\ddagger}$	8.36							320
D/D	1.91	(0.62)		12.92				2.21
D/D/D	1.04	(0.48)		4.49	(0.27)		14.77	0.52
L	8.31		[11.71]					16.40
L/L	3.12	(0.67)	[5.39]	13.81		[0.05]		0.49
$2\sigma X$	±0.22	(±0.05)	[±0.92]	±0.31		[±0.04]		
Dansylated CK in 6 M GDN/HCl								
D	7.94							160
D/D	2.78	(0.60)		11.94				1.20
D/D/D	1.89	(0.27)		3.75	(0.67)		12.52	0.60
L	7.81		[7.07]					7.15
L/L	4.01	(0.68)	[3.06]	12.56		[0.05]		0.81
$2\sigma X^2$	±0.19	(±0.04)	[±0.90]	±0.51		[±0.81]		
Dansylated AK								
D	10.38							229
D/D	2.72	(0.60)		14.58				2.70
D/D/D	1.33	(0.39)		5.20	(0.28)		16.75	0.59
L	9.89		[10.32]					22.72
L/L	4.30	(0.64)	[4.49]	15.20		[1.15]		1.10
$2\sigma X^2$	±0.12	(±0.05)	[±1.2]	±0.50		[±0.70]		
Dansylated AK in 6 M GDN/HCl								
D	5.20							290
D/D	1.59	(0.70)		8.70				3.07
D/D/D	0.73	(0.52)		3.97	(0.35)		11.06	0.52
L	5.81							21.20
L/L	2.21	(0.78)	[3.49]	8.83		[0.89]		0.90
$2\sigma X^2$	±0.16	(±0.08)	[±1.0]	±0.31		[±0.09]		

*Results listed here were from data set obtained with a Schott VG10 bandpass filter Schott American Glass & Scientific Products, Inc. centered at 540 nm. $^{\ddagger}D$, *D/D*, and *D/D/D* are single, double and triple exponential decays, respectively; *L and L/L* are unimodal and bimodal Lorentzian distributions, respectively.

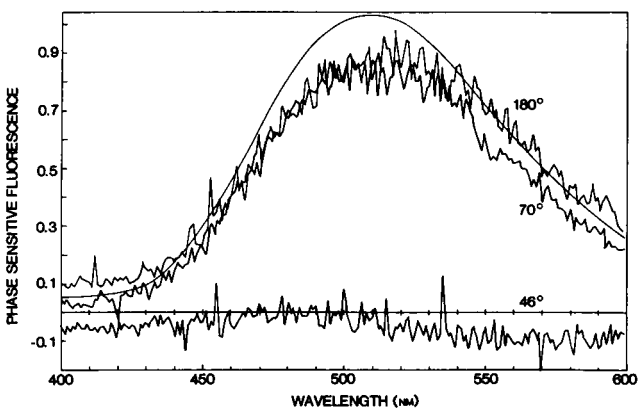

FIGURE 3 Analogue phase resolved spectra of doubly dansylated CK. Sample is described in Fig. 1. Phase-resolved spectra were obtained with glycogen as a zero lifetime reference. Phase angles used during spectral acquisition in the dynamic mode are given next to the spectrum illustrated. The smooth curve represents the steady-state spectrum.

1). However, even in the presumed denatured state, two distributed or three discrete lifetimes still best describe the phase/modulation data. Denatured CK displays an increase in the central lifetime of the shorter lifetime component but no significant change in the longer decay component in the Lorentzian model. The distribution is narrowed for the shorter component, but the fractions of the lifetime associated components are unchanged. The effect of denaturant on dansylated CK is more apparent from comparison of the discrete decays laws. Exposure to 6 M GDN/HCl results in a decrease in the lifetimes of components two and three and an increase in the population of the second lifetime component. The discrete model form of the decay law for dansylated CK and AK exposed to denaturant reveal significant differences in the shortest and longest lifetimes and a greater population for the middle lifetime component of dansylated CK. A more profound effect of denaturant on AK is observed from the distributed decay law as seen in the

decrease in the distributed lifetime of the longer component.

Fluorescence quenching

Quenching of dansylated CK fluorescence intensity by increasing acrylamide concentrations exhibits a downward curvature of the Stern-Volmer plot (Fig. 4 *a*), characteristic of the occurrence of multiple fluorophores in environments of different solvent accessibility. Replotting of the results according to

$$F_o/\Delta F = 1/(fK[Q]) + 1/f, \qquad (4)$$

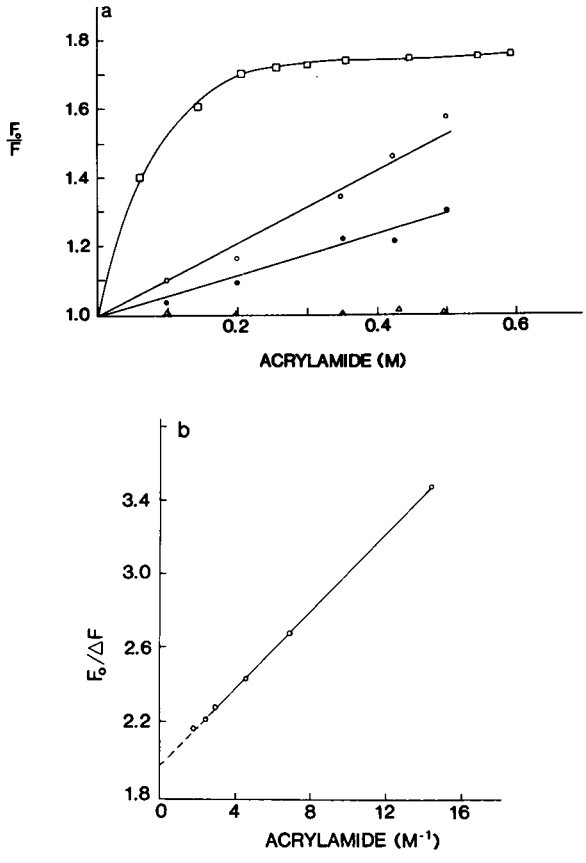

FIGURE 4 (*a*) Stern-Volmer plot for the quenching of doubly dansylated CK by acrylamide. Sample contained 12.5 μM protein in 0.1 M potassium phosphate buffer, pH 8.0, and 2 mM dithiothreitol. Fluorescence was titrated by addition of aliquots from a 6 M solution and intensity adjusted for dilution. Excitation at 325 nm and emission at 510 nm. Acrylamide did not absorb light of 325 nm wavelength obviating correction for "inner filter effects" up to the highest concentrations examined. Lifetimes were determined as described in the text on the same preparation described above at the same concentration. (□), F_o/F; (○), $(\tau_o/\tau)_1$; (●), $(\tau_o/\tau)_2$; (△), $(\tau_o/\tau)_3$. (*b*) Fluorescence quenching of doubly dansylated CK by acrylamide plotted according to Eq. 4. Data the same as that illustrated in Fig. 5 *a* for the steady-state measurements.

where F_o is the fluorescence in the absence of quencher, ΔF the fluorescence increase in the presence of quencher of concentration Q, f the fraction accessible to quenching, and K the quench constant (Fig. 4 *b*) (15), indicates that 51% of the total fluorescence intensity is not quenched. Also, the lifetime of the longest component is unchanged upon titration with acrylamide. The two shorter lifetimes both display a small degree of dynamic quenching as judged from the decreases in lifetimes accompanying addition of acrylamide. The quench constants are 2.83 and 2.16 M for the shortest and medium value lifetime components in the triple decay law. Experiments with potassium iodide did not reveal any quenching of dansylated CK, either by lifetime or steady-state measurements.

Energy transfer

Owing to spectral overlap, the tryptophan emission and dansyl excitation are useful for resonance energy transfer analysis. Results from donor quenching experiments (Fig. 5, Eq. 1) and sensitized emission (Fig. 6, Eq. 3) indicate energy transfer of 38 and 35%, respectively. For the sensitized emission experiments, dansyllysine in propanol, matched in absorbance at 340 nm with the dansylated protein, was used in the calculations as the acceptor moiety in the absence of donor. Table 2 summarizes the decay laws for the intrinsic fluorescence in the absence and presence of the dansyl moiety. Comparison of the weighted average of the lifetimes of the tryptophans of unlabeled and dansylated CK showed a transfer efficiency of 31%, according to Eq. 2.

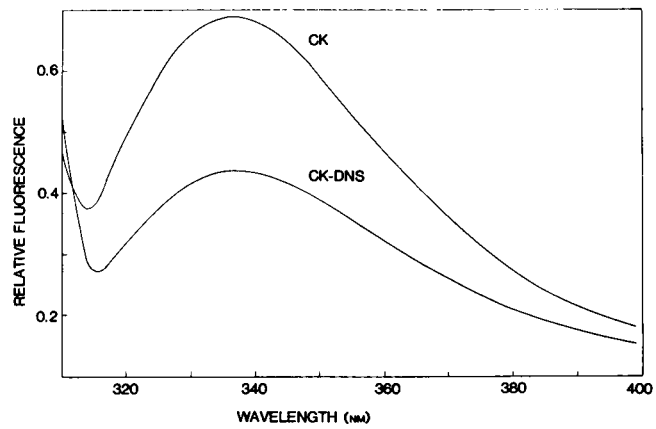

FIGURE 5 Energy transfer determined from donor quenching. Samples described in Fig. 1 were matched in absorbance at 280 nm after correction for the contribution of the dansyl moiety, with unlabeled CK. Spectra illustrated are both excitation and emission corrected. Energy transfer calculated according to Eq. 2.

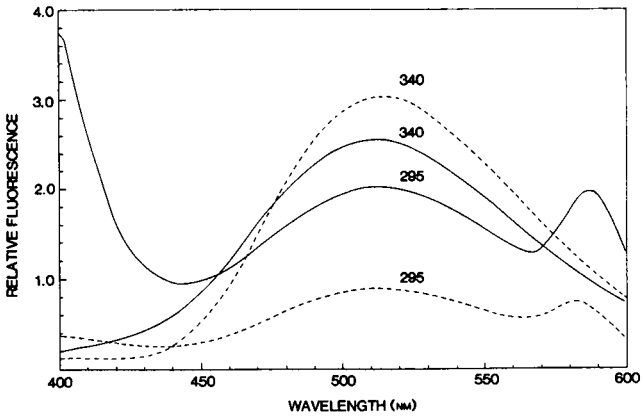

FIGURE 6 Energy transfer determined from sensitized acceptor emission. Samples of dansyllysine (acceptor in the absence of donor) and dansylated CK (donor/acceptor) were matched in absorbance at the absorption maximum of the acceptor (dansyl moiety). Solid lines are doubly-dansylated CK and dashed lines are dansyllysine in propanol excited at the indicated wavelength. Absorbance of acceptor (dansyllysine) at 295 nm and absorbance of donor (CK) were used with the spectra illustrated and Eq. 4 to calculate the sensitized emission. All spectra are emission corrected.

DISCUSSION

Specific dansylation of both reactive lysines of CK and the single reactive lysine of AK are possible only after complete protection of the highly reactive cysteines with sodium tetrathionate (5). The original description of the preparation of dansylated CK and AK (5) is accompanied by a rigorous identification of the site specificity and stoichiometry of dansylation. A similar preparation of dansylated CK was used to demonstrate the essential role of lysine in the phosphotransferase mechanism and it was shown that introduction of the dansyl group does not alter the binding site domain conformation (6). This conclusion is based on the observation that the dansylated derivative had little effect on the dissociation constants of substrates from the ternary complex.

The dependence of the fluorescence lifetime of the dansyl moiety upon solvent polarity is characteristic of environmentally sensitive fluorophores: the amide derivative of dansyl chloride, 1-(dimethylamino)-5-naphthalenesulfonamide, has a lifetime of 3.3 ns in water and 17.1 ns in pure 2-propanol (17). This model fluorophore also exhibits a dependence of lifetime upon dioxane concentration similar to the one observed for dansyllysine in propanol solutions in the present study. Dansylleucine displays a similar lifetime dependence upon solvent (18). The solvent sensitivity of the fluorescence intensity of 1-dimethylaminonaphthalene-5-sulfonate is also evident from the quantum yields reported to be 0.37 in aqueous solvent and 0.72 in 95% ethanol (19).

TABLE 2 **Intrinsic fluorescence lifetimes of creatine kinase and dansylated creatine kinase**

Sample	τ_1	$(A)^*$	τ_2	χ^2	Weighted lifetime‡
CK	0.587	(0.60)	3.6	1.02	(1.76)
CK/DNS	0.228	(0.66)	3.5	0.92	(1.34)

* Lifetime associated intensity. ‡ $\tau = \Sigma\tau_i A_i / \Sigma A_i$.

Although the data sets were overdetermined at least three times and in some instances five times, there does not appear to be sufficient information to resolve the question of discrete vs. distributed lifetimes. Multiexponential decays have also been observed for dansylated histone subunits (18) and it was shown that the discrete model is a better description of the data than the bimodal distributions. In a study of tryptophan fluorescence of sarcoplasmic reticulum ATPase, Gryczynski and co-workers (20) found the data could be fit equally well by a three component discrete model and a bimodal distribution, although they note that the distribution model is helpful in recognizing the complexities of fluorescence decay.

The decay laws, whether discrete or bimodal for the lifetimes of doubly dansylated CK indicate that the two ligands are exposed to solvent of highly differing polarities. The simplest model is to assign each lifetime to the microenvironment around the dansyl moieties, one on each subunit, with the active sites having different solvent characteristics. This would support the proposed asymmetric nature of the subunit arrangement (3, 21). However, the finding that monomeric, singly labeled AK also exhibits a multiexponential decay would indicate that the bimodality or three discrete lifetimes, in doubly dansylated CK need not reflect a dimeric state with disparate intramolecular dansylation sites. An alternate suggestion is that both AK and CK exist in multiple conformational states, one (or a set) reflecting a polar environment and the other (or another set) a nonpolar environment around the fluorophore. The actual structural difference between the two states, although reflected by large differences in lifetimes may represent rather minor shifts in conformation and it remains possible that within a conformational state, the two subunits are asymmetrically arranged.

The results of the acrylamide quenching of doubly dansylated CK are characteristic of proteins containing fluorophores with different degrees of solvent accessibility (15). The titration of a maximum of ~50% of intensity with acrylamide, suggests a large fraction of the total fluorescence is not accessible to the quencher. The dynamic component of the quenching, as revealed from

lifetime measurements is atypically small. The longest-lived component shows no change in lifetime, suggesting that the fluorophore displaying the lifetime of 14 ns, is not accessible to the quencher. The two shorter lifetimes do exhibit dynamic quenching, but the quench constants were very small and close enough to each other to suggest they may represent quenching of the same species (indicating that it is these two lifetimes which are located in the shorter lifetime component of the bimodal continuum). The fact that the magnitude of quenching, judged from intensity, is much greater than that from the lifetimes, leads to the unusual conclusion that the dansyl moiety is mainly statically quenched when conjugated to CK. A model might include a high affinity site on CK for acrylamide, adjacent to the dansyl moiety. In this system, it appears dynamic quenching is a minor pathway for deactivation of the excited state.

The occurrence of multiple conformational states would be consistent with the following previous observations: (*a*) differential reactivity of presumably homologous sites on both subunits, proposed to indicate asymmetry in subunit arrangement (2, 3), is equally well explained by the occurrence of two conformeric dimers. (*b*) Cooperativity in substrate binding (1) would be observed if substrate induced a conformational isomerization between dimeric protein states.[2] (*c*) Fast and slow folding steps in renaturation (22) would be observed if two dimeric forms with different folding kinetics occurred, e.g., one with a rate limiting proline isomerization. (*d*) Isolated, but unextracted myofibrils will still bind exogenous CK into the M-line, in spite of the presence of a large excess pool of soluble CK in vivo (4). Conformational states may be compartment specific. (*e*) Different probes when bound to the identical site, reveal different environmental polarities (23).

A nonequivalence of energy transfer efficiency derived from steady-state and time resolved methods is indicative of polypeptide chain flexibility (24). The finding that the efficiency of energy transfer measured from steady state and lifetime determinations were close in a study of the distance between the active sites of muscle CK (25), suggests that the overall dimer has little conformational flexibility. In the present instance, the two steady state and lifetime derived values for the transfer efficiency between the dansyl moieties and tryptophan (eight per dimer) (1) are close enough to also suggest only limited motion between the donors and the acceptor. Additional characterization of overall flexibility would be derived from results of dynamic anisotropy studies.

The limited differences in the decay law between dansylated CK in buffer and dansylated CK in 6 M GDN/HCl, suggest either that the dansyl moiety is not undergoing environmental changes during unfolding or that the dansyl associated domain is resistant to denaturation. The first suggestion would seem unlikely because the long lifetime component reflects a nonpolar environment, which should display a shorter lifetime in a more polar solvent accompanying protein unfolding as is observed with dansylated AK. The possibility of resistance to denaturation is supported by the very limited spectral differences observed between dansylated CK in buffer and in 8 M urea (7) and the kinetic evidence that ordered structure is retained by CK in 4 M GDN/HCl (12). Structural homology between guanidine and the CK substrate creatine may be a factor in domain stability.

The results provide evidence for heterogeneity in the fluorescence decay of dansylated AK and CK consistent with the existence of at least two conformeric states. The less populated conformer is characterized by a highly hydrophobic environment for the active site resident dansyl moiety which is inaccessible to acrylamide quenching. The other conformer possesses the dansyl ligand in a more hydrophilic environment. For CK, one or both conformers may possess domains resistant to complete denaturation.

The soluble and myofibrillar M-line associated creatine kinase activities are the same protein (26). The latter is sufficiently active to supply all the ATP required for myosin activity during contraction (27). It may be the case that isozyme specific compartmentation is based on conformer specificity, and if this specificity extends to substrate binding, then new regulatory aspects of the ATP generating mechanism may be worth consideration.

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[2]In preliminary studies, we have observed that the presence of the catalytic dead-end complex composed of creatine, magnesium chloride and ADP, consistently increases the fractional contribution of the lower lifetime component to the total intensity amplitude, but is without significant effect on the lifetimes or distributions.

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