Resonance Energy Transfer between the Active Sites of Rabbit Muscle Creatine Kinase: Analysis by Steady-State and Time-Resolved Fluorescence†

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ABSTRACT: Resonance energy transfer between the reactive thiols of rabbit muscle creatine kinase was evaluated. The reactive thiols are located at the active site, one occurring on each subunit of the dimeric protein that is known to be a constituent of the M-line structure of the myofibril. Transfer efficiency was evaluated from energy donor quenching of fluorescence by steady-state and phase-modulation lifetime measurements and determination of sensitized emission of the acceptor. Several sulfhydryl-specific donor fluorophores were used including 5-[[[(iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic acid, 7-(dimethylamino)-3-(4-maleimidylphenyl)-4-methylcoumarin, and 2-[4-(iodoacetamido)anilino]naphthalene-6-sulfonic acid (IAANS). Energy transfer acceptors included 5-(iodoacetamido)fluorescein and the nonfluorescent dye [4-[[4-(dimethylamino)phenyl]azo]phenyl]iodoacetamide. In order to prepare the necessary homodimer labeled with both donor and acceptor, advantage was taken of the biphasic reaction between creatine kinase and IAANS. In some instances, donor/acceptor hybrids were prepared by denaturation/renaturation procedures, and possible deviations from expected hybridization stoichiometry were considered. Disproportionation of singly labeled dimers (to unlabeled and doubly labeled dimers) was not observed when the brain isozyme of creatine kinase was used to trap dissociated dye-conjugated or unlabeled muscle-type subunits of creatine kinase. From studies of five different donor/acceptor combinations, the efficiency of energy transfer was found to occur over a range of 5–14%, indicating that the reactive thiols are well separated. Overlap integrals and quantum yields were evaluated, and estimates of the range of orientation factor were obtained to determine a range for the distance between the active sites of creatine kinase. When the ranges are overlapped, a limited distance of 48.6–60.4 Å is obtained. Phase-resolved anisotropy measurements yield a rotational correlation time of approximately 40–50 ns, with several of the dye-conjugated preparations also exhibiting fast motions consistent with probe rotation or segmental flexibility. The site–site distance of the muscle isozyme is compared to the previously determined shorter distance for the hybrid, myocardial-specific isozyme creatine kinase MB [Grossman, S. H. (1983) *Biochemistry 22*, 5369–5375], suggesting a potential difference in subunit arrangement which could account for isozyme-specific compartmentation. The site–site distance also is considered with respect to possible interaction between the active site of creatine kinase bound to the M-line structure and the rod portion of myosin.

Cytoplasmic creatine kinase occurs in three dimeric forms, designated by their tissue distribution: CK-MM,[1] muscle; CK-BB, brain; CK-MB, myocardium (Watts 1973). A small fraction of the MM isozyme protein is identified as the m-bridge structure of the myofibril M-line (Wallimann et al., 1978). Association between CK-MM and the rod portion of myosin has been demonstrated in vitro (Hauk & Putnam, 1973; Mani & Kay, 1976). It is apparent that the MM isozyme possesses structural features distinct from the BB homodimer and hybrid protein, which are involved in interdigitation with M-line structures.

Among the many similar physical and catalytic features of the CK isozymes are the molecular weights (Watts, 1973), frictional ratios (Yue et al., 1967, 1968), and rotational relaxation times (Grossman, 1983a). Similar catalytic mechanisms include the inhibitory effects of the pseudo-transition-state complex containing MgADP–creatine–nitrate anion (Milner-White & Watts, 1971; Grossman & Garcia-Rubio, 1987). The mechanism and kinetics of folding during renaturation/reassociation of denatured CK-MM and CK-BB are very similar (Grossman et al., 1981, 1986). Amino acid sequence analysis shows 80% homology between the B and the M subunit (Pickering et al., 1985). On the other hand, the M subunit is richer in basic residues (Watts, 1973), reflected in the different isoelectric points (Grossman & Mollo, 1979), and the homodimers are antigenically non-cross-reactive (Roberts et al., 1976). The question arises regarding the nature of the conformational, steric, or surface charge features that form the basis of specificity in isozyme compartmentation and antigenicity yet are generated from relatively homologous sequences that do not produce dramatically different higher order structures. In the absence of high-resolution crystallographic data, it would be useful to obtain a quantitative measure and comparison of some specific site–site distances among the isozymes.

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[1] Abbreviations: CK, creatine kinase; CK-MM, muscle-type isozyme of CK; CK-BB, brain-type isozyme of CK; CK-MB, hybrid, myocardial-type isozyme of CK; IAEDANS, 5-[[[(iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic acid (AED moiety); CPM, 7-(diethylamino)-3-(4-maleimidylphenyl)-4-methylcoumarin (CPM moiety); IAANS, 2-[4-(iodoacetamido)anilino]naphthalene-6-sulfonic acid (AANS moiety); IAF, 5-(iodoacetamido)fluorescein (AF moiety); DABIA, [4-[[4-(dimethylamino)phenyl]azo]phenyl]iodoacetamide (DAB moiety); CK–AED, CK–AANS, CK–CPM, CK–AF, and CK–DAB, muscle isozyme of creatine kinase doubly conjugated with each of the dye moieties represented above (singly dye-conjugated derivatives are similarly abbreviated but indicated as such in the text); DTT, dithiothreitol; POPOP, 1,4-bis(5-phenyloxazol-2-yl)benzene; Xd, donor-labeled subunit; Xa, acceptor-labeled subunit.

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With the aim of gaining a measure of subunit arrangement, we used Förster energy transfer analysis to determine an approximate active site–active site distance of 27–52 Å for isozyme MB (Grossman, 1983b). Each subunit contains a highly reactive sulfuryl at the active site, which is readily derivatized by a variety of fluorescent dyes (Haugland, 1975; Price, 1979; Grossman, 1983b). The heterodimer was chosen for the original study because a suitable energy donor/acceptor hybrid could be prepared by denaturation/renaturaton of a mixture of acceptor- and donor-labeled homodimers MM and BB. The same procedure cannot be used for the MM isozyme since techniques to separate the products after hybridization are not available. However, there is evidence that CK-MM possesses asymmetrically arranged subunits (Degani & Degani, 1980) and exhibits biphasic kinetics in reaction with certain fluorophores (Price, 1979). These properties could be exploited in preparing a donor/acceptor hybrid for energy transfer analysis, but there is also evidence that, once formed, singly labeled dimers disproportionate into a mixture of doubly labeled and unlabeled dimers (Price, 1979).

No single method was deemed satisfactory for preparation of donor/acceptor-conjugated CK-MM, and consequently, several experimental approaches to preparation and evaluation of the transfer efficiency were investigated. Fluorescence lifetime measurements by phase-modulation procedures (Lakowicz, 1983) were used in mixtures containing donor/donor-, acceptor/acceptor-, and donor/acceptor-labeled CK. By utilizing the differential reactivity of the two sulfhydryls toward certain reagents, it was possible to evaluate energy transfer by steady-state measurements. Finally, a preparation was obtained by hybridization procedures that was suitable for analysis of sensitized acceptor emission. Steady-state polarization and time-resolved anisotropy measurements were used to obtain parameters applicable to estimating an appropriate donor/acceptor orientation factor and overall protein rotational rates. The results of the study are considered in terms of localization of the active sites, isozyme structural differences, and potential interaction between CK-MM and myosin.

Experimental Procedures

Rabbit muscle creatine kinase was purchased from Sigma Chemical Co. and, when necessary, further purified by hydroxyapatite chromatography (Grossman & Mollo, 1979). Brain CK (CK-BB) was isolated from frozen rabbit brains by the same procedure used to isolate the isozyme from monkey brain (Grossman & Mollo, 1979). Protein concentration was determined by the Coomassie Blue dye binding assay (Bradford, 1976) or by measurement of the absorbance at 280 nm where $A(1\%,1\text{ cm}) = 8.8$ (Kuby & Noltmann, 1962) for CK-MM. Enzyme activity was determined with the assay described by Rosalki (1967). Protein purity was assessed by polyacrylamide gel electrophoresis according to the procedure of Laemmli (1970). Creatine kinase isozymes MM and BB were hybridized by addition of a final concentration of 8 M urea to the protein in buffer, incubation for 1 h at room temperature, and then renaturation by dialysis against the same buffer without denaturant. Isozyme hybridization products were separated and detected by agarose thin-film electrophoresis and enzyme-coupled assay as described previously (Grossman et al., 1981).

Fluorescence dyes (Molecular Probes, Junction City, OR) were conjugated with CK-MM in 0.1 M K-TES, pH 7.0, or 0.1 M potassium phosphate, pH 8.0. After reaction, samples were filtered through a small column of Sephadex G-25 and dialyzed against the reaction buffer, supplemented with 2 mM DTT. In experiments designed to react the fluorophores at the two reactive cysteines, a typical protocol involved addition of a 10-fold molar excess of the dye to 30 μM protein in 2.0 mL of buffer. After gentle rotation at 4 °C in the dark for 24 h, the reaction was quenched by addition of DTT to a final concentration of 5 mM; the sample was then centrifuged and applied to the gel filtration column. This procedure was used to double conjugate CK-MM with IAF, IAEDANS, and IAANS. Single conjugation of CK with IAANS was carried out as described by Haugland (1975), except protein was reacted with a molar equivalent of dye.

The insoluble dye CPM was first adsorbed onto cellulose (10% by weight) and reacted as described for the soluble dyes (Grossman, 1983b). The nonfluorescent, sulfhydryl-reactive reagent DABIA was first dissolved in a minimum of dimethylformamide and then added to the CK solution, not exceeding 10% by volume. A preparation containing one DAB per CK was obtained if the reaction was stopped after 16 h by the addition of DTT. For labeling of both reactive sulfhydryl groups by DABIA, the reaction required 36 h. Reaction kinetics between CK and DABIA could be accelerated by addition of a greater fold excess of DABIA, but this led to significant protein precipitation.

Reactive sulfhydryl content of each preparation was determined with Ellman's reagent (Habeeb, 1972). After dialysis to remove the liberated nitrothiobenzoate, the samples were again reacted with each of the dyes. Since the reaction of the insoluble dyes with the nitrothiobenzoate derivative of CK produced considerable precipitated protein, subsequent analysis was not possible. In these cases, particularly with DABIA, a CK-MM derivative with thiols blocked by use of potassium tetrathionite was used (Kassab et al., 1968; Grossman, 1984).

The degree of reaction was determined from the absorption spectrum of each dye conjugate with reference to extinction coefficients for each dye derivitized with *N*-acetylcysteine. Conjugates between the dyes and *N*-acetylcysteine were prepared by reaction of dye with a 10-fold molar excess of *N*-acetylcysteine at pH 8.0 in 0.1 M potassium phosphate buffer. For the insoluble dyes, dilute stock solutons were first prepared in 2-propanol and then diluted 1:1 (v/v) in buffer. Absorption spectra and enzyme assays were determined with a Gilford Model 250 scanning spectrophotometer.

Fluorescence measurements were performed with an SLM 48000 spectrofluorometer. For steady-state intensity and spectral measurements, the emission monochromator was used. For fluorescence polarization, lifetime, or dynamic anisotropy measurements, the appropriate emission cutoff filters (Corning) were used in place of the monochromator. Polarization and anisotropy measurements were made in the T-format with dual-emission photomultipliers. The cuvette chamber was maintained at 25 °C with circulating thermostated water and continuously purged with dry nitrogen during data acquisition. All spectra illustrated were excitation corrected by reference to rhodamine as a quantum counter and emission corrected by use of factors supplied by the instrument manufacturer. Steady-state polarization measurements, performed with the polarizer rotation sequence described previously (Grossman, 1983b), are the average of 12 determinations with a standard deviation of less than 1%.

The lifetime reference standard was ethanolic POPOP (τ = 1.32 ns) (Lakowicz et al., 1981). The data acquisition interval and number of acquisitions at each frequency depended upon the intensity of the sample. The minimum used was an average of five measurements of 15 s each. In some instances, especially for dynamic anisotropy measurements, it was necessary to average eight readings of 100 s each. Data

that exhibited a standard deviation above 0.5 and 0.005 for the phase shift and demodulation, respectively, were discarded. A typical lifetime evaluation consisted of data acquisition from 2 to 120 MHz or until a phase angle shift of 70° was achieved. A minimum of 20 frequencies was used within the frequency domain. One-term, two-term, and three-term decay laws were probed to match the best fit of the experimental data. The decay law providing the lowest match at the assumed standard deviation of 0.5 for the phase shift and of 0.005 for the demodulation was selected. When the χ^2 could not be reduced to below 1, it was concluded that the heterogeneity of the preparation was too great for generation of an acceptable decay law or that the resident fitting routines were inadequate. In some cases, where two decay laws were close, simulation of the data was performed by using a wider range with more inclusive frequencies. The fit that most closely resembled one of the decay laws was chosen as the best model for the experimental data.

Time-resolved anisotropy measurements were made as described by Lakowicz et al. (1984) except that for the fitting routines the estimated Δ-phase error was 0.5°. The decay laws consulted included single, double and triple correlation times. In general, the values of the limiting anisotropy and the correlation times were floated, or several fixed values of rotation were input and solved for additional rotations and limiting anisotropy. The lifetime used was usually the weighted average of the determined lifetimes, and these almost always provided the best fit, when the other parameters were minimized. In every case, the phase and modulation data obtained experimentally were compared with simulated data, over a wider and more inclusive frequency range, and generated from application of the decay equations after input of lifetime, limiting anisotropy, and one, two, or three rotational correlation times.

Results

Stoichiometry of Dye Conjugation. The extinction coefficients (M^{-1} cm^{-1}) derived for *N*-acetylcysteinyl derivatives were as follows: CPM, 28 500; DAB, 20 900; AF, 48 900; AANS, 18 100; AED, 6200. The values obtained for reaction of CK with excess reagent as described under Experimental Procedures give a stoichiometry of 1.82 (±0.09) to 1.92 (±0.09) dye molecules bound per molecule of dimeric CK. Samples of CK reacted with Ellman's reagent (Habeeb, 1972) (and dialyzed free of 2-nitro-5-thiobenzoate) and then treated with each of the dyes failed to exhibit greater than 0.15 mol of dye/mol of dimeric CK, after correction for the absorbance of the nitrothiobenzoate conjugate. Doubly dye-conjugated CK, regardless of the dye, after dialysis to remove DTT, was observed to react with no more than 0.1 mol of Ellman's reagent/mol of protein. In each case, the doubly dye-conjugated derivatives exhibited less than 9% of the activity of an equivalent concentration of native CK.

Hybridization Reactions. Donor/donor dye-conjugated CK was hybridized with either native CK or acceptor/acceptor dye-conjugated CK. To determine the stoichiometry of product formation, experiments were performed to separate the products of the hybridizations. Efforts to resolve the products of the hybridization mixture of CK–CPM and unlabeled CK by ion-exchange chromatography (Grossman, 1983b), agarose gel electrophoresis (Grossman et al., 1981), affinity chromatography (Easterday & Easterday, 1974), and preparative isoelectric focusing were unsuccessful. In the denaturation/renaturation experiments on the doubly dye-conjugated CK derivatives, all the preparations exhibited near-complete reversibility to the pretreated derivatives (Price, 1979; Grossman, 1983b) except CK–AANS, which was not used in denaturation/renaturation experments.

Table I: Overlap Integrals (J) of Donor/Acceptor Pairs, Quantum Yield of Donors (Q_0), and Half-Transfer Distance (R_0), Assuming Orientation Factors of 0.66, 0.05, and 3.0

donor/acceptor	$J \times 10^{15}$ (M^{-1} cm^3)	Q_0	$R_0(0.66)$ (Å)	$R_0(0.05)$ (Å)	$R_0(3.0)$ (Å)
AED/DAB	67	0.30	39.0	25.3	50.2
CPM/DAB	93	0.14	37.4	24.4	49.2
CPM/AF	102	0.14	38.0	24.4	47.4
AANS/AF	121	0.08	34.5	22.4	44.4
AANS/DAB	81	0.08	32.2	20.9	42.9

In order to achieve an estimate of the degree of hybridization, the MM isozyme was hybridized with CK-BB doubly conjugated with IAF (CK–AF) (Grossman, 1981). When separated by agarose thin-film electrophoresis, the products of the denaturation/renaturation exhibited a fluorescein moiety intensity of 59% and 41% for the MB and BB fractions, respectively, and when resolved on DEAE-Sephadex chromatography (Grossman, 1983b), they exhibited 91% MM, 45% MB, and 0% BB activities when compared to the hybridization production of similarly treated but unlabeled CK-MM and CK-BB. The approximate intensity ratio of 3:2:0 and retention of most catalytic activity by unlabeled monomers in the dimer suggest that singly labeled dimers do form in near-expected stoichiometry and retain native structure.

Evaluation of the Overlap Integral (J), Quantum Yield (Q_0), and Critical Transfer Distance (R_0). Overlap integrals (Table I) were evaluated from the acceptor absorbance spectrum and corrected donor emission spectrum of dye-conjugated CK preparations (Forster, 1965), according to

$$J = \int \frac{\epsilon_\lambda F_\lambda \lambda^4 \, d\lambda}{F_\lambda \, d\lambda} \quad (1)$$

where ϵ is the extinction coefficient of the acceptor and F is the corrected relative fluorescence of the donor.

The quantum yield of each of the donor fluorophore–CK conjugates was measured by reference to the quantum yield of quinine bisulfate (QBS) in 1 N sulfuric acid according to

$$Q_0(\text{donor}) = Q(\text{QBS})A(\text{QBS}) \times \text{area(donor)}/[A(\text{donor}) \times \text{area(QBS)}] \quad (2)$$

where A is the absorbance at 350 nm and area refers to the area under the corrected emission spectrum of a sample excited at 350 nm. The area measurements were made directly from the recorded spectra with the available integration programs for the fluorometer. The value of $Q = 0.54$ (Adams et al., 1977) for the quantum yield of quinine bisulfate, as determined from optoacoustic spectrometry measurements, was used in the calculation.

The distance across which energy transfer is 50% efficient is determined from

$$R_0 = (J\kappa^2 Q_0 n^{-4})^{1/6}(9.79 \times 10^3) \quad (3)$$

where n is the refractive index, estimated to be 1.33, and κ^2 is the orientation factor, relating the direction of the dipole vectors for the donor and acceptor ligands.

Table I lists the calculated values for the half-transfer distance on the basis of orientation factors of 0.05, 3, and 0.66. The first two represent extreme cases where the dipoles are fixed, without significant motion, whereas the 0.66 value represents complete reorientation of both donor and acceptor within the excited-state lifetime. Information regarding donor and acceptor rotation can be obtained from static and dynamic anisotropy measurements as described below.

Energy Transfer. (*a*) *Steady-State Measurement of Donor Quenching.* CK–CPM was hybridized with (1) unlabeled CK and (2) CK–DAB. The products of these hybridizations are (1) doubly and singly conjugated CK–CPM and unlabeled CK and (2) doubly conjugated CK–CPM and CK–DAB and the hybrid CK–CPM/DAB. Ideally, the fluorescence emission of CK–CPM/DAB should be compared with the emission of CK–CPM (singly labeled) from the spectra obtained by excitation at the maximum for CPM. Since it is not possible to separate the products of interest from the other dimerizations occurring during hybridization, e.g., re-formed parent dimers, energy transfer can only be estimated from a comparison of the total emission of each of the hybridization products, adjusted for the contribution of the donor/donor conjugate. Assuming that the denaturation/renaturation leads to all three products in the stoichiometry expected for association between protomers with equal affinity for each other, the data can be corrected for the emission due to the doubly dye-conjugated CK–CPM present in both mixtures.[2] By use of

$$E = 1 - F_{d/a}/F_d \quad (4)$$

where F is the corrected fluorescence emission by the donor/acceptor pair (d/a) and by the donor conjugated protein (d), the results obtained indicate that energy transfer is 5% efficient.

(*b*) *Donor Quenching during Reaction with Acceptor.* Kinetics of donor quenching upon addition of acceptor was used to determine energy transfer. The IAANS fluorophore was chosen as donor because the significant biphasic reaction kinetics (Haugland, 1975; Price, 1979), permits labeling of a single subunit. When the reaction reaches a point when one

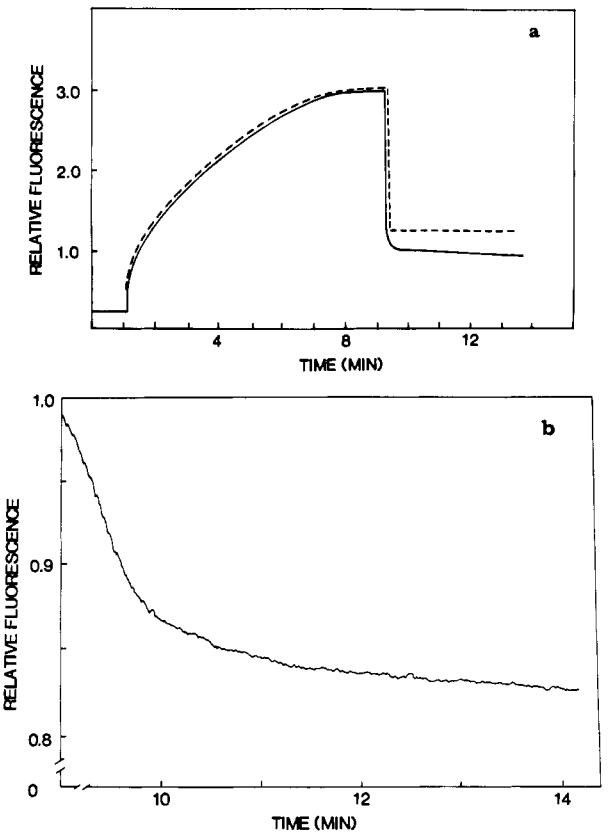

FIGURE 1: (a) Reaction kinetics between CK, IAANS, and IAF. Samples of 2.0 μM IAANS in 2.0 mL of 0.1 M K-TES, pH 7.0, were equilibrated at 25 °C in a cuvette and monitored for fluorescence at 350-nm excitation and 470-nm emission. After 1 min, 0.2 mL of CK was added to a final concentration of 1.8 μM (dimers). Fluorescence was recorded an additional 8 min followed by the addition of 0.01 mL of IAF solution (—) or 0.005 mL of DTT–AF solution (– –) to a final concentration of 10 μM. (b) Enlargement of the relative fluorescence change immediately after the addition of the excess IAF to the reaction product of IAANS and CK. Previous studies had indicated that 8 min of incubation of equimolar concentrations of CK and IAANS at pH 7.0 produced a labeling of one sulfhydryl per molecule of CK.

Table II: Phase-Resolved Lifetimes of CK–AED and CK–AED/DAB

sample	τ_1 (ns) [(%)]	τ_2 (ns) [(%)]	$\langle\tau\rangle^a$
CK–AED	9.82 (20)	18.68 (80)	17.57
CK–AED denatured/renatured	9.91 (18)	18.57 (82)	17.66
CK–AED/DAB[b]	5.53 (5)	17.05 (95)	16.86
N-acetylcysteinyl-AED	10.20 (100)		

[a] $\langle\tau\rangle = \sum\alpha_i\tau_i^2/\sum\alpha_i\tau_i$. [b] Doubly dye-conjugated CK–AED (2.0 mL, 10 μM) was mixed with excess doubly dye-conjugated CK–DAB (1.0 mL, 60 μM) and treated with sufficient urea to bring the final denaturant concentration to 8 M. After incubation at 4 °C for 60 min, the sample was dialyzed against 0.1 M K-TES, pH 7.0, and 2 mM DTT for 48 h with two changes of 200 mL of buffer over that period. It can be shown[3] that under these conditions, the hybrid contains 75% of the total donor-labeled subunit. Lifetimes were determined by using 31 frequencies in the domain of 5–110 MHz. Values for χ^2 were 0.44 or less. Excitation, 340 nm; emission, 470-nm cutoff filter.

AANS reacts with CK, addition of excess IAF results in an instantaneous decrease in emission, followed by an additional but small time-dependent loss of fluorescence (Figure 1a). The instantaneous decay results from attenuation of excitation light by absorbance due to AF as judged from control experiments with nonreactive DTT/AF conjugate and the known absorptive properties of fluorescein at 350 nm. One interpretation of the slower and smaller decay, expanded in Figure 1b, is that it is due to donor quenching by reaction of IAF at

[2] A general expression for determining the molar relationship of the products dependent upon association constants K_0 (homodimer) and K_t (heterodimer), where $n = K_0/K_t$, is $(n+1)$XdXd + $(n+1)$XaXa → (n)XdXd + (n)XaXa + 2XdXa. (a) Starting with equal initial concentrations and assuming equal promoter affinities ($n = 1$)

$$2XdXd + 2XaXa \rightarrow XdXd + XaXa + 2XdXa \quad (A)$$

$$2XdXd + 2XX \rightarrow XdXd + XX + 2XdX \quad (B)$$

If the spectra of the mixture in eq A are 5% quenched compared to the spectra of the mixture in eq B, then the transfer efficiency is 10%. (b) Starting with equal initial concentratioins but unequal promoter affinities (e.g., $n = 4$)

$$5XdXd + 5XaXa \rightarrow 4XdXd + 4XaXa + 2XdXa \quad (C)$$

$$5XdXd + 5XX \rightarrow 4XdXd + 4XX + 2XdX \quad (D)$$

If the spectra of the mixture in eq C are 5% quenched compared to the spectra of the mixture in eq D, then the transfer efficiency is 25% (FU = fluorescence units). [4XdXd + 2XdX yields 100 FU and 4XdXd + 2XdXa yields 95 FU. In both cases 80 FU is contributed by XdXd, leaving 15 FU of fluorescence contributed by XdXa and 20 FU contributed XdX. The transfer efficiency is $E = (1 - 15/20) \times 100$.] (c) Combining possibilities described above in which the affinities in the homodimers are greater than the affinities of the hybrids, $n = 1$ for the doubly conjugated hybrids and $n = 4$ for the singly labelled hybrid

$$2XdXd + 2XaXa \rightarrow XdXd + XaXa + 2XdXa \quad (E)$$

$$5XdXd + 5XX \rightarrow 4XdXd + 4XX + 2XdX \quad (F)$$

If the spectra of the mixture in eq E is 5% quenched compared to the spectra of the mixture in eq F, then the transfer efficiency is 58%. [XdXd + 2XdXa yields 95 FU and 4XdXd + 2XdXa yields 100 FU. In eq E, 47.5 FU is contributed by XdXd, leaving 47.5 FU of fluorescence contributed by XdXa. In eq F, 80 FU is contributed by XdXd, leaving 20 FU contributed by XdXa. The transfer efficiency is $E = (1 - 20/47.5) \times 100$.]

the other reactive sulfhydryl. The possibility of a second-site reaction inducing a conformational change at the AANS site is unlikely since the spectral characteristics, most importantly, the emission maximum of the highly environmentally sensitive AANS moiety, are the same for the doubly and singly dye-conjugated proteins. Experiments also showed the reaction product, iodide anion, did not the cause donor quenching. From these results, it appears that AF at one sulfhydryl results in quenching of approximately 10% of the donor emission. Long-term monitoring of product formation did not exhibit an increase in donor fluorescence, as might be expected if reaction of the acceptor was followed by a disproportionation of subunits.

The kinetics of the labeling reaction between singly derivatized DAB–CK and IAANS showed only a small difference between the emission intensity of the singly labeled CK–AANS after 1 h of reaction in the presence and absence of previously bound DAB, which was used to calculate a transfer efficiency of 5%.

(*c*) *Lifetime Measurements.* Energy transfer was detected with lifetime measurements on a hybrid prepared from doubly conjugated CK–AED and CK–DAB. An excess of the acceptor (CK–DAB) was used in the hybridization in order to minimize re-formation of the parent CK–AED in the denaturation/renaturation cycle. The results of the lifetime measurements (Table II) indicate a small decrease in the weighted lifetime of the CK–AED compared to those of the hybridization products, which are predominantly the energy transfer hybrid CK–AED/DAB and lesser amounts of CK–AED and the nonfluorescent conjugate CK–DAB. The transfer efficiency calculated according to

$$E = 1 - \tau_{d/a}/\tau_d \quad (5)$$

where $\tau_{d/a}$ is the weighted lifetime of the hybridization products and τ_d is the weighted lifetime of the donor before hybridization is 5%. If only the longer lifetimes are compared, the energy transfer efficiency is approximately 10%.

(*d*) *Acceptor Sensitized Emission.* Preparation of the donor/acceptor CPM/AF conjugate was performed by denaturation/renaturation of doubly dye-conjugated CK–AF with a large excess of CK–CPM to minimize the re-formed double conjugate of AF–CK. Similar denaturation/renaturation cycles were performed between unlabeled CK and doubly dye-conjugated CK–CPM or CK–AF. Absorption spectra of the three hybridizations are shown in Figure 2a. Figure 2b compares the emission spectrum of the mixture obtained after hybridizing CK–CPM with excess unlabeled CK with the spectrum obtained from hybridizing CK–CPM with CK–AF. The difference spectrum (Figure 2c) is characteristic of the emission spectrum for the AF moiety. This is compared in Figure 2c with the spectrum obtained from the hybridization of CK–AF with excess unlabeled CK, excited at the excitation of the donor CPM. The small difference suggests limited sensitized emission. Direct excitation spectra of the products of the hybridizations of CK–AF with CK–CPM and of CK–AF with CK (unlabeled) at the excitation for the AF moiety are virtually identical (Figure 2d). Finally, Figure 2e compares the emission of the CK–CPM and CK–AF hybridization products using the donor excitation wavelength with the emission of CK–AF hybridized with CK (unlabeled) using the acceptor excitation wavelength. These data are used to calculate the transfer efficiency according to

$$E = (A_a^{\lambda_a}/A_d^{\lambda_d})(F_{da}^{\lambda_d}/F_a^{\lambda_a}) \quad (6)$$

when there is no absorbance by the acceptor in the excitation band of the donor. When the acceptor does absorb at the excitation of the donor

$$E = (A_a^{\lambda_a}/A_d^{\lambda_a})[(F_{da}^{\lambda_d}/F_a^{\lambda_a}) - 1] \quad (7)$$

is used. Excitation at 400 nm was chosen because of the low fluorescein absorption and direct fluorescence excitation at that wavelength. Again the calculations are based on the expected product stoichiometry obtained from mixing the dimers in a 10 to 1 ratio and adjusting for the fluorescence of the emission due to the presence of doubly dye-conjugated CK–CPM.[3] The transfer efficiencies were 13% and 14% from eq 6 and 7, respectively.

The transfer efficiencies determined by the methods of steady-state donor quenching, lifetime donor quenching, and steady-state sensitized acceptor emission are summarized in Table III.

Static and Dynamic Anisotropy. From the ratio of the steady-state anisotropy for the fluorophore covalently bound in the protein r_0 to the fundamental anisotropy r_f for the free fluorophore, some limiting estimates of the orientation factor are derived (Dale et al., 1979). The square of this ratio is designated the axial depolarization factor, and the smaller the value, the less the uncertainty in κ^2. By utilization of the values obtained previously (Hudson & Weber, 1973; Grossman, 1983) and data collected in the present study (Figure 3), the axial depolarization values were determined, and the resulting

Table III: Transfer Efficiencies and Distance between the Active Sites of CK-MM

donor/acceptor	method	transfer efficiency, E	transfer distance (κ^2) (Å)		
			R(0.66)	R(0.05)	R(3)
CPM/DAB	donor quenching[a]	0.05	61.1	39.9	80.4
CPM/AF	sensitized emission[b]	0.13	52.2	34.1	65.1
AED/DAB	lifetime[c]	0.10	56.3	36.5	72.4
AANS/AF	donor quenching	0.10	49.8	32.3	64.1
AANS/DAB	donor quenching	0.05	52.6	34.2	70.1

[a] From eq 4. [b] From eq 6. [c] From eq 5.

Table IV: Limiting (r_0) and Fundamental (r_f) Anisotropies, Axial Depolarization Factors $\langle d_x \rangle$, Estimated Range of Orientation Factor (κ^2), and Actual Transfer Distance (R) for Fluorescent Donor/Acceptor Derivatives of CK-MM[a]

donor/acceptor	r_0	r_f	$\langle d_x \rangle^b$	κ^2 range[c]	R range (Å)
CPM/AF				0.10–3.0	36.8–65.1
donor	0.324	0.327	0.98		
acceptor	0.290	0.364	0.64		
AANS/AF				0.28–2.1	43.1–60.4
donor	0.230	0.319	0.52		
acceptor	0.290	0.364	0.64		

[a] Angular displacements were CPM, 4.5°; AANS, 25.6°; and AF, 21.6° on the basis of steady-state polarization measurements and eq 8. [b] $\langle d_a \rangle^2 = \langle d_x \rangle = r_0/r_f$, where $\langle d_a \rangle$ is the observed depolarization. [c] From Dale et al. (1979).

[3] It is possible to compute the expected product ratio if initial molar concentrations of starting dimers are varied prior to dissociation:

$$(m + 1)\text{XdXd} + [m(m + 1)]\text{XaXa} \rightarrow \text{XdXd} + (m^2)\text{XaXa} + (2m)\text{XdXd}$$

where m = acceptor/donor ratio and $m/(m + 1)$ = fraction of the total donor participating in the donor/acceptor hybrid. For example, for an acceptor/donor ratio of 3 (4XdXd + 12XaXa → XdXd + 9XaXa + 6XdXa) the fraction of total donor in the donor/acceptor is 0.75.

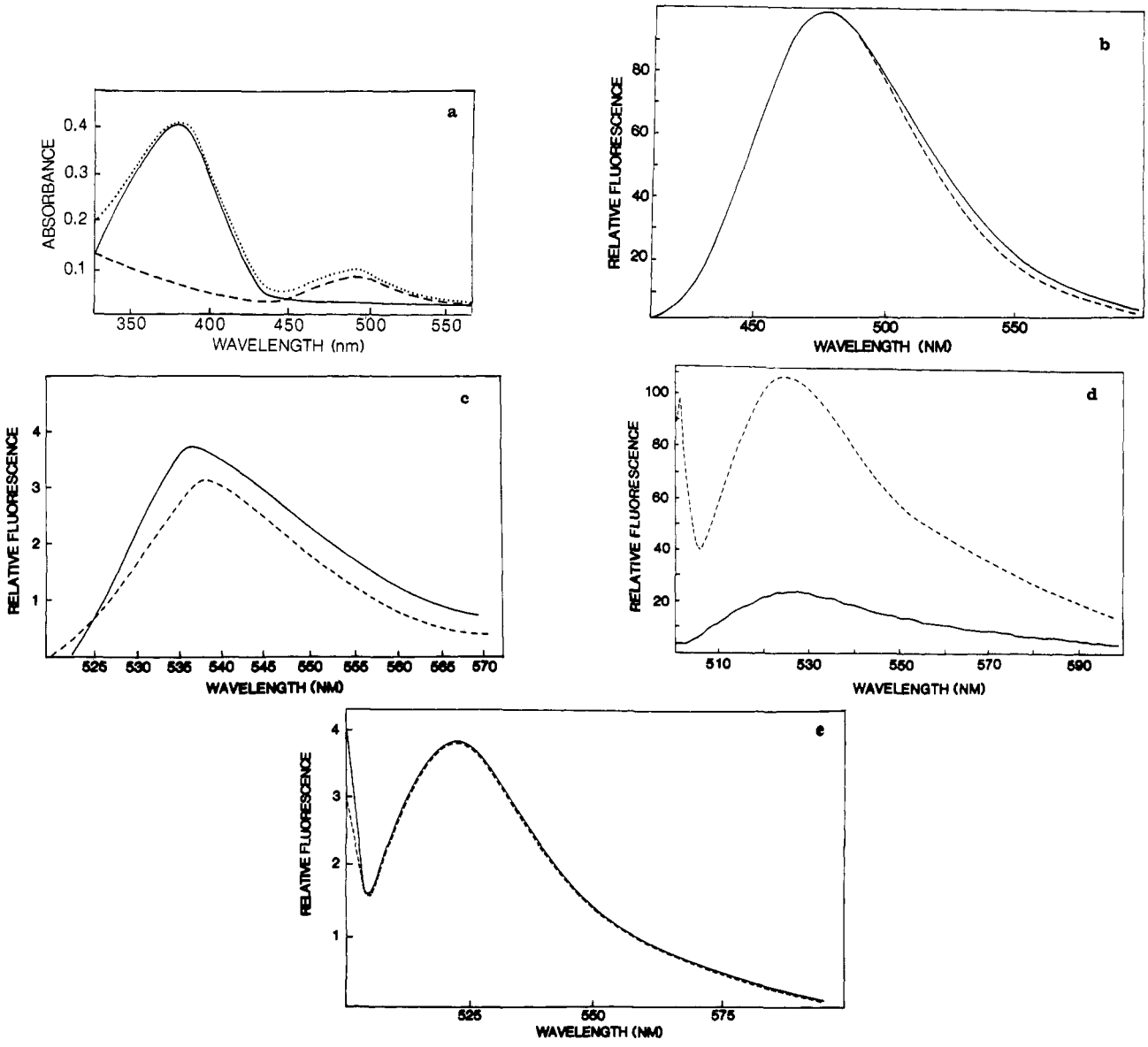

FIGURE 2: Determination of transfer efficiency by sensitized acceptor emission. Initial concentrations of unlabeled and labeled CK stock solutions were 5 μM. Samples of CK–AF and CK–CPM were mixed in a molar ratio of 1 to 10. Similarly, CK–AF and unlabeled CK were mixed in a molar ratio of 1 to 10, and finally CK and CK–CPM were mixed in a molar ratio of 1 to 10. Samples were denatured by addition of urea to 8 M concentration and incubated for 30 min (total volume 1.5 mL), followed by renaturation by dialysis against 0.1 M K-TES, pH 8.0, and 2 mM DTT. Samples were centrifuged and diluted to identical protein concentrations, followed by determination of absorption spectra (a) [CK–AF (– –); CK–CPM (—); CK–CPM/AF (···)] and fluorescence (excitation 400 nm) spectra (b) [CK–CPM (– –); CK–CPM/AF (—)]. Panel c illustrates the difference spectrum (– –) obtained from the two spectra in panel b and compares it to the spectrum obtained by direct excitation of CK–AF at 400 nm (—). Panel d illustrates the fluorescence spectrum of CK–AF excited at 495 nm (– –) compared to the fluorescence spectrum of the sensitized emission of CK–CPM/AF excited at 400 nm (—). Panel e shows that the CK–AF and CK–CPM/AF preparations exhibit virtually identical fluorescence when excited at 495 nm, the excitation maximum for AF.

ranges of the orientation factors were obtained from the contour plots in Dale et al. (1979). The results provide some improvement in the estimate of R as shown in Table IV. From

$$r/r_0 = (3\cos^2\theta - 1)/2 \quad (8)$$

one can estimate the angular displacement of the fluorophore when attached to the protein. The highest value obtained was for protein-bound AED with a value of 27.2°. In this study, transfer depolarizations were not evaluated, and it was not possible to apply this analysis to the nonfluorescent CK–DAB-containing hybrids.

Phase-resolved limiting anisotropies and rotational correlation times are listed in Table V. For CK–AED and CK–AED/DAB, a considerable amplitude for a short correlation time component is evident, revealing rotational motion associated with the fluorophore. A short rotational correlation time is also observed for AF- and CPM-conjugated CK. Only the AANS-derivatized CK conjugate exhibits a single rotational correlation time, at 32 ns, which is likely to reflect macromolecular motion (see Discussion). These results should be

Table V: Limiting Anisotropies (r_0) and Rotational Correlation Times (θ) Obtained for Dye Conjugates of CK Determined from Time-Resolved Anisotropy Measurements

sample	τ (ns)	r_0	θ_1 (ns) [(%)]	θ_2 (ns) [(%)]
CK–AANS	3.2	0.305		32.0 (100)
CK–AF	4.1	0.333	0.4 (23)	26.8 (77)
CK–CPM	3.9	0.277	12.1 (39)	38.0 (61)
CK–CPM/DAB	3.8	0.292	9.0 (32)	40.0 (68)
CK–AED	17.6	0.187	2.4 (25)	51.3 (75)
CK–AED/DAB	16.8	0.200	2.1 (37)	45.1 (63)
CK–AED (6 M Gdn–HCl)	12.2	0.208	0.6 (71)	4.1 (29)
N-acetylcysteinyl-AED	6.8	0.122	0.3 (100)	

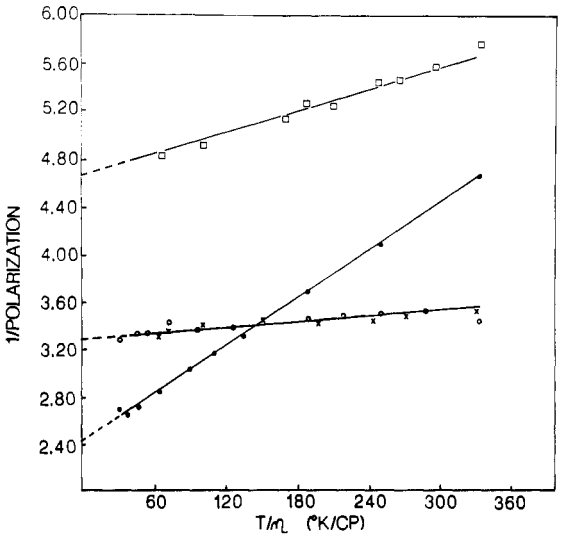

FIGURE 3: Fluorescence polarization of IAANS, CK–AANS, and CK–AED. Samples analyzed consisted of IAANS (0.4 μM), CK–AANS (0.2 μM), and CK–AED (0.2 μM) in 0.1 M K-TES, pH 7.0, with 2 mM DTT. Viscosity (25 °C) was varied by the addition of enzyme-grade sucrose (○, □, ●) or glycerol (×). Additional details are given in the text. CK–AED (□); CK–AANS (○, ×); IAANS (●).

contrasted with the amplitudes and rotational correlation times of denatured CK–AED, which lacks the long correlation time but does exhibit a major short component (600 ps) which is indicative of nearly complete freedom of rotation of the probe as judged by the value of 300 ps obtained for the amino acid derivatized AED conjugate.

Disproportionation. The occurrence of disproportionation of dimeric CK labeled with a single bulky fluorophore into doubly conjugated dimers and unlabeled dimers has been reported to occur within 30 min of the labeling reaction (Price, 1979), the likely mechanism being dissociation and reassociation. To test this, each isozyme separately or mixed was treated with sufficient IAANS to label only a single subunit. If any dissociation/reassociation of the active subunits occurred, even if only one were still active (Dreyfus et al., 1968), it would be indicated by the appearance of some active CK-MB. The results showed no CK-MB activity, suggesting that during and after the reaction period an exchange of subunits did not occur. This experiment was repeated 16 times under a wide range of conditions including protein concentration, time of incubation, and buffer pH, and no hybrid was ever detected. Furthermore, when the electrophoresis plate was not stained for activity but instead examined directly with long-wavelength ultraviolet light, faint bands were detected at the positions appropriate for CK-MM and CK-BB but not for CK-MB.

In another approach, after initiation of reaction between of CK-MM and IAANS and at intervals thereafter, aliquots were removed, and denatured CK-BB (in 8 M urea) was added. The final urea concentration was less than 1 M, which is known to permit refolding of the denatured protein (Grossman et al., 1981). In this instance too, active CK-MM and reactivated CK-BB, but no hybrid, were detected.

Discussion

In view of the difficulties in differential labeling of potentially homologous sites, we have utilized four different methods to assess energy transfer and have analyzed five different donor/acceptor pairs. In no instance did we observe the complete absence of transfer nor greater than 14% transfer. In a limited study, Haugland (1975) reported 25% energy transfer efficiency between the reactive sulfhydryls of CK-MM, while Bickerstaff and Price (1978) were unable to cross-link the reactive thiols with a series of bifunctional reagents. The active sites are well separated with the narrowest range for the actual transfer distance, obtained from a wide range of potential orientation factors and delimitation from measurement of depolarization factors, being 48.6–60.4 Å.

The dyes used in this study have been shown previously to be useful as energy transfer donors and acceptors (Price, 1979; Lin & Dowben, 1983; Dalbey et al., 1983; Grossman, 1983b; Chantler & Tao, 1986). The CK–AANS conjugate was chosen for its potential as a CK derivative conjugated at only one of the two reactive thiols. DAB is particularly valuable because of its energy acceptor properties without fluorescence emission (Luedtke et al., 1981).

Two experimental problems with the present approach are (a) the possibility of lower than predicted concentrations of the donor/acceptor hybrid in the preparations involving dissociation/reassociation and (b) possible disproportionation (Price, 1979). Since the actual degree of hybridization in the denaturation/renaturation experiments is not known, it was possible that if the donor/acceptor hybrid represented only a small component of the mixture, then after correction for the donor/donor homodimer fluorescence the observed transfer would be an underestimate of the actual value. However, in the experiments not involving dissociation/reassociation, CK–AANS reacted with IAF and CK–DAB reacted with IAANS, only 10% and 5% transfer efficiencies, respectively, were observed, similar to the low values obtained by the other procedures employed. It is unlikely that disproportionation occurred *during* preparation (reaction time: 8 min) since under identical conditions disproportionation was not observed with subunit hybridization or exchange techniques. Had the ratio of donor/donor to acceptor/acceptor to donor/acceptor been as high as 2:2:1 rather than the 1:1:2, which represents a 75% decrease in the amount of donor-labeled subunit participating in the hybrid, the transfer efficiency would still only be 25%. Furthermore, a stable CK–CPM/AF hybrid and singly labeled CK–CPM and CK–AF of CK–MB were prepared in high yield previously (Grossman, 1983b). Dreyfus et al. (1968) also showed that an active and stable CK-MB could be prepared by hybridizing CK-MM with CK-BB previously fully inactivated by iodoacetamide. In addition, neither the kinetics nor the model proposed by Degani and Degani (1980) to account for slow and fast thiol reaction kinetics of CK-MM, which they proposed to be a dimer of asymmetrically arranged subunits, is consistent with the occurrence of disproportionation. We did note that reaction of singly labeled CK-AANS with a second IAANS was considerably slower than the rate of the first labeling reaction, although not as slow as might be expected from the kinetics of double labeling with excess IAANS (Price, 1979). We also noted that when IAF was reacted with singly labeled CK–AANS and produced donor quenching, extended incubation did not exhibit decay to the original fluorescence intensity, as expected if disproportionation occurred. Finally, we attempted to trap a dissociated M-subunit (with B-subunit), expected to occur during reaction between CK and sufficient IAANS to single label CK, but were unable to detect any evidence of the putative CK-MB hybrid. These results indicate that in the experimental procedures we have used the subunits of CK do remain associated after derivatization with the donor/acceptor fluorophores under consideration.

Biexponential decays are the most suitable decay laws for IAANS or IAEDANS when dissolved in 20% buffered pro-

panol, suggesting that the two lifetimes exhibited by the derivatized proteins represent intinsic properties of the dyes. In a preliminary study, we have determined that doubly and singly dye-conjugated CK–AANS do not exhibit significantly different lifetimes, but both do show biexponential decays. The values for the lifetimes obtained are generally reproducible to between 0.05 and 0.1 ns in repeated evaluations on the same sample. However, while shortening of the lifetimes, either weight averaged or the longer component, and donor quenching are small due to the presence of the acceptor, they are significant and yield well above the 2% limit of usefulness of Förster energy transfer analysis.

The rotational correlation time calculated for CK if it were a sphere is about 22 ns [rotational relaxation time of 66 ns (Grossman, 1983a)]. Lakowicz et al. (1983) derived an expected value of CK of 43.5 ns using a frictional coefficient of 2 but, using the intrinsic fluorescence, found experimentally a value of 13.8 ns. The difference is attributed to the substantial segmental flexibility of the domains of the tryptophans, of which there are eight (Watts, 1973). Several of the dye-conjugated proteins studied in the present report exhibited long rotational correlation times consistent with the calculated value. The shorter values for θ observed for CK–AF and CK–AANS may indicate some additional shorter (anisotropic) rotation(s) which could not be resolved or reflect the inadequacy of the relationship between τ and θ. In the case of CK–CPM, the rotational correlation time is at the expected value for the protein as whole, but the shorter value, 12.1 ns, is too long to represent the probe motion itself and may indicate additional modes of anisotropic rotation or large domain flexibility. Several of the dye conjugates also exhibit short rotational correlation times of considerable amplitude and, as shown for CK–AED by using CK-AED in denaturant and the model AED compound, represent probe motion.

In a study of CPM/AF-labeled CK-MB, energy transfer was approximately 0.55, and the estimated transfer distance was 27–52 Å. The energy transfer efficiency for the same pair of conjugates on the MM isozyme was 0.13. Assuming that the orientation factors for the dye on both proteins are similar, the active sites of CK-MM are likely to be further apart than those on CK-MB. The narrowest range obtained in the present study is 49–60 Å. Since the B- and M-subunits exhibit identical frictional ratios (Yue et al., 1967, 1968) and nearly superimposable circular dichroic spectra (unpublihed determination), it is likely that the subunits have similar three-dimensional characteristics. To account for the difference in the active site distances between isozymes MM and MB, it could be suggested that the association of subunits in the heterodimer is significantly more asymmetric. This may be a factor to account for the finding that CK-MM but not CK-MB is found bound to the M-line structure of the myofibril (Wallimann et al., 1977).

The unit crystal dimensions for CK-MM are a = 47 Å, b = 86 Å, and c = 125 Å (McPherson, 1973). The frictional ratio is 1.2 with a major/minor axis of 4.4 for an assumed prolate ellipsoid (Yue et al., 1967). The proposed structure of the dimer is visualized by Watts (1973) to be like two cigar-shaped subunits laying side by side. Given the distance between the reactive thiols, the active sites are proposed to be situated relatively close to the opposite ends of the dimer. If we consider a distance of about 55 Å (the midpoint of the maximally delimited range) and the proposed subunit asymmetry of the homodimer CK-MM (Degani & Degani, 1980) not to have a pronounced effect on subunit conformational similarity, then the active centers are proposed to be localized approximately 35 Å [(125 − 55)/2] from the end of each subunit.

Wallimann et al. (1983, 1984) have concluded that the myofibrillar M-line m-bridges are creatine kinase possessing activity sufficient to replenish most or all of the ATP hydrolyzed by myosin ATPase during contraction. Luther and Squire (1978) show the m-bridges to lie perpendicular to the rod portion of myosin, although proximity to the hinge regions is unclear. If creatine kinase binds to myosin in a perpendicular fashion, surface to surface, the active site would be about 35 Å from the rod filament of myosin. This distance would be too great to allow conformational changes in CK brought on by catalysis (Watts, 1973) to affect myosin function or flexibility, unless the structures were extremely tightly coupled. On the other hand, if a segment of the CK prolate ellipsoid (m-bridge) were interdigitated *into* the M-line, then substrate binding could affect the conformation of surrounding structures. Although the exact site of interaction and the components necessary to observe interaction between CK and myosin are unclear from in vitro studies (Woodhead & Lowey, 1983), a very early observation (Yagi & Mase, 1962) showed that CK-MM does inhibit myosin ATPase activity.

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Resolution of Tyrosyl and Tryptophyl Fluorescence Emission from Subtilisins†

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ABSTRACT: Subtilisin Carlsberg is an exception to Teale's general rule [Teale, F. W. J. (1960) *Biochem. J. 76*, 381-388] that in proteins which contain both tyrosine and tryptophan residues the predominant contribution to the emission is from tryptophan [Longworth, J. W. (1971) in *Excited States of Proteins and Nucleic Acids* (Steiner, R. F., & Weinryb, I., Eds.) pp 319-484, Plenum Press, New York]. The tyrosyl and tryptophyl fluorescence contributions of underivatized subtilisin Carlsberg and the homologous enzyme subtilisin BPN′ were resolved in this study. Steady-state and picosecond time-resolved measurements over the whole emission spectrum were performed at different excitation wavelengths. Data were analyzed by using global techniques, and associated spectra of the exponential decay components were derived. Samples of subtilisin Carlsberg purified by a novel method and free of autolysis products were found to emit from *both* tyrosine and tryptophan at an excitation wavelength of 295 nm. There was evidence for a small tyrosine contribution in the emission from subtilisin BPN′ excited at 295 nm and in the emission from subtilisin Carlsberg excited at 300 nm. Careful purification of the enzymes is necessary in order to eliminate fluorescence from autolysis products.

Subtilisins are serine proteases produced by various species of *Bacillus*. Subtilisin Carlsberg (from *Bacillus licheniformis*) and subtilisin BPN′ (from *Bacillus amyloliquefaciens*) have been well studied (Markland & Smith, 1971), and their crystal structures are known at high resolution (Bode et al., 1987; Bott et al., 1988). The Carlsberg enzyme is used as a protein-digesting agent in detergents and is produced in greater quantities than any other industrial enzyme (Aunstrup et al., 1979). The BPN′ enzyme has been studied as a model system for protein engineering (Wells & Estell, 1988).

Subtilisin Carlsberg contains 274 amino acid residues differing from subtilisin BPN′ at 84 positions and by a deletion at Pro^{56} of BPN′. The former includes 1 tryptophan (Trp^{113}) and 13 tyrosine residues while the latter contains 3 tryptophan residues (Trp^{113}, Trp^{106}, and Trp^{241}) and 10 tyrosine residues (Markland & Smith, 1971). There is considerable sequence and structural homology between the two proteins which can assist in the interpretation of their fluorescence properties. Subtilisin Carlsberg displays unusual fluorescence characteristics. It is an exception to the general rule that in proteins which contain both tyrosine and tryptophan residues the overwhelming contribution to the emission is from tryptophan (Longworth, 1971). There has been some disagreement in the literature over the extent of tryptophan emission in subtilisin Carlsberg (Schlessinger et al., 1975; Brown et al., 1977). Since intrinsic fluorescence has been used to assess structural (Vaz & Schoellmann, 1976; Boteva et al., 1988) and dynamic (Bayley et al., 1987) properties of subtilisins, it is important that this is clarified. Fluorescence studies have been further complicated by the presence of autolysis products which may also contribute to the emission.

The purpose of this investigation was to resolve the tyrosyl and tryptophyl contributions to the emission of subtilisin Carlsberg and BPN′. This was achieved by combining the

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