Friedberger, E., & Brossa, G. A. (1912) *Z. Immunitätsforsch. Exp. Ther., 1 15*, 506.
Galli-Valerio, B., & Bornand, M. (1916) *Z. Immunitätsforsch. Exp. Ther., 1 25*, 154.
Goldstein, I. J., & Hayes, C. E. (1978) *Adv. Carbohydr. Chem. Biochem. 35*, 127.
Gueugnot, J. (1980) Doctorat d'Etat ès Sciences Pharmaceutiques, Clermont I.
Guillot, J., & Coulet, M. (1974) *Rev. Fr. Transfus. 17*, 49.
Karlsson, C., Davies, H., Ohman, J., & Andersson, U. B. (1973) *LKB Application Note 75*, LKB Produkter AB, Stockholm.
Kochibe, N., & Furukawa, K. (1980) *Biochemistry 19*, 2841.
Kogure, T. (1973) *J. Jpn. Soc. Transfus. 19*, 127.
Kogure, T. (1975) *Vox Sang. 29*, 221.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Mäkelä, O. (1957) *Ann. Med. Exp. Biol. Fenn., Suppl. 35*.
Mäkelä, J., Mäkelä, P., & Krupe, M. (1959) *Z. Immunitätsforsch. Exp. Ther., 1 117*, 220.
Merland, A. M., & Coulet, M. (1961) *C. R. Seances Soc. Biol. Ses Fil. 155*, 2248.
Periera, M. E. A., Kisalius, E. C., Gruezo, F., & Kabat, E. A. (1978) *Arch. Biochem. Biophys. 185*, 108.
Raszeja, S. (1958) *Z. Aerztl. Fortbild. 14*, 801.
Rosenfield, R. E., & Haber, G. V. (1965) in *Technicon Symposium*, New York, p 515.
Silicani, V., Nicoli, R. M., Ranque, J., & Battaglini, P. F. (1962) *Bull. Soc. Pharm. Marseille 11*, 1.
Tetry, A., Sutton, E., & Moullec, J. (1954) *C. R. Hebd. Seances Acad. Sci. 238*, 277.
Voak, D., & Lodge, T. W. (1971) *Vox Sang. 20*, 36.

Resonance Energy Transfer between the Active Sites of Myocardial-Type Creatine Kinase (Isozyme MB)†

Steven H. Grossman

ABSTRACT: The single reactive sulfhydryl group, located in the active site of each subunit of dimeric creatine kinase from rabbit muscle (isozyme MM), was selectively labeled with 3-(4-maleimidylphenyl)-7-(diethylamino)-4-methylcoumarin (CPM). Isozyme BB, purified to homogeneity from rabbit brain, was conjugated with the sulfhydryl-specific reagent 5′-(iodoacetamido)fluorescein (5′-IAF). Spectral analyses demonstrated that 1.8 mol of CPM and 1.9 mol of 5′-IAF had reacted per mol of protein. Labeled isozymes were combined, denatured in 8 M urea, and renatured by dialysis, producing the parent labeled homodimers and forming the heterolabeled hybrid dimer, creatine kinase MB. Similar hybridizations were performed to prepare singly labeled hybrids, starting with labeled and unlabeled homodimers. The hybrid isozymes were isolated by ion-exchange chromatography, and spectral analyses of singly labeled heterodimers revealed overlap between the absorption spectrum of MB labeled with acetamidofluorescein on the B subunit and the corrected fluorescence emission spectrum of MB labeled with CPM on the M subunit. Analyses included evaluation of the quantum yield of the CPM-labeled hybrid, estimation of the range of the orientation factor K^2 from fluorescence polarization and anisotropy studies, and determination of J, the spectral overlap integral for the fluorescence donor (CPM-labeled MB) and acceptor (acetamidofluorescein-labeled MB). Results of these experiments permitted an estimation of R_0, the distance between the donor and the acceptor at which energy transfer is 50% efficient. Comparison of the relative fluorescence of the donor in the presence (heterolabeled hybrid) and absence (hybrid conjugated with CPM on the M subunit) of the acceptor or determination of the normalized sensitization of the acceptor fluorescence led to an evaluation of the transfer efficiency and the actual transfer distance of between 27 and 52 Å. The kinetics of quenching of CPM fluorescence, during hybridization with CPM-labeled MM and acetamidofluorescein-labeled BB, compared to hybridization with unlabeled BB, suggest that substantial refolding of denatured subunits precedes reassociation.

Cytoplasmic creatine kinase occurs in three tissue-specific dimeric forms. The muscle isozyme, designated CK-MM,[1] has been extensively characterized both kinetically (Morrison & James, 1965; Jacobs & Kuby, 1970) and structurally (Yue et al., 1967). It is composed of two similar, if not identical, subunits of M_r 41 000, with an axial ratio of 4 for an assumed anhydrous prolate ellipsoid. Watts (1973) has likened the overall conformation to two cigar-shaped subunits positioned side by side, a conceptualization supported by X-ray crystallographic studies (McPherson, 1973). However, subunit association does not appear to be an obligatory requirement for catalytic activity (Grossman et al., 1981).

While the brain-type isozyme, CK-BB, is clearly distinct from the muscle type by the criteria of amino acid content (Watts, 1973; Grossman & Mollo, 1979), electrophoretic

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[1] Abbreviations: CK, creatine kinase; MM, the muscle type isozyme of creatine kinase; BB, the brain type isozyme; MB, the hybrid, myocardial isozyme; 5′-IAF, 5′-(iodoacetamido)fluorescein; CPM, 3-(4-maleimidylphenyl)-7-(diethylamino)-4-methylcoumarin; M_c, M subunit conjugated with CPM; B_f, B subunit conjugated with acetamidofluorescein; M_cM_c, B_fB_f, M_cB_f, M_cB, and MB_f, dye-conjugated dimers; DDT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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properties (Eppenberger et al., 1967; Grossman & Mollo, 1979), and antigenicity (Bulcke & Sherwin, 1969; Grossman & Mollo, 1979), their overall conformations are similar (Yue et al., 1968). Kinetic evidence (Jacobs & Kuby, 1970) and studies of fluorescence polarization (Grossman, 1983) suggest that CK-BB undergoes more profound conformational changes than CK-MM upon interaction with substrate and is a looser or more flexible protein. The third isozymic form, myocardial-specific, heterodimeric CK-MB, while composed of a B and M subunit, behaves more like CK-BB with respect to isoelectric point and heat inactivation (Grossman & Mollo, 1979; Morel-Deletraz, 1979) but remains the least well-characterized isozymic form. Association of the subunits in the heterodimer could result in significant conformational changes or generation of asymmetry.

Each subunit of creatine kinase possesses a catalytic site in which is located a highly reactive sulfhydryl residue (Mahowald et al., 1962). This site is ideally suited for labeling with an appropriate fluorescence energy donor or acceptor (Stryer, 1978). Subsequent analysis for energy transfer would provide data for the estimation of the intramolecular distance between the active centers. Here we report the preparation of doubly dye conjugated isozyme MB and analysis of resonance energy transfer. An evaluation of the distance between the active site of the subunits of the hybrid isozyme provides information concerning their relative position in the dimer as well as the possibility of interaction between the active sites. The results may be compared with the studies of Haugland (1975) which suggest that the active sites of CK-MM are separated by a distance of at least 30 Å.

Experimental Procedures

Creatine kinase from rabbit muscle was purchased from Sigma. Rabbit brain creatine kinase was purified by the method reported for the isolation of monkey brain isozyme (Grossman & Mollo, 1979). Both preparations were judged homogeneous by displaying a single Coomassie blue staining band following electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate (Weber & Osborn, 1969). Enzyme activity was measured spectrophotometrically by the procedure of Rosalki (1967). Protein concentration was determined by the fluorescamine assay (Böhlen et al., 1973) or the Coomassie blue procedure of Bradford (1976). Reaction with Ellman's reagent was performed as described by Habeeb (1972). The method of Trudinger (1961) was used to prepare *S*-sulfenylsulfonate-derivitized proteins.

Isozyme MM (5 mg/mL) was reacted with CPM (Molecular Probes, Plano, TX), adsorbed to cellulose (5 mg of dye/200 mg of cellulose) in a total volume of 2.0 mL of 0.1 M sodium phosphate, pH 8.0. After stirring gently in the dark for 48 h at 4 °C, the suspension was filtered through glass wool and applied to a column (2 × 50 cm) of Sephadex G-25, equilibrated with reaction buffer. The column was eluted with the same buffer, and eluant fractions that emerged in the void volume and were fluorescent under UV light (366 nm) were combined and exhaustively dialyzed against 0.1 M sodium phosphate buffer, pH 8.0, and 2 mM DTT.

Isozyme BB (5 mg/mL) in 2.0 mL of 0.1 M sodium phosphate, pH 8.0, was treated with 5 mg of 5′-(iodoacetamido)fluorescein (5′-IAF) (Molecular Probes) at 4 °C in the dark. After 6 h, the reaction mixture was subjected to gel filtration and dialysis as described above.

Three hybridizations were carried out: M_cM_c with B_fB_f, M_cM_c with BB, and MM with B_fB_f. Muscle isozyme (1.2 mg in 2 mL) was mixed with brain isozyme (1.5 mg in 2 mL) and dialyzed at 4 °C against 500 mL of 8 M urea in 0.1 M sodium phosphate, pH 8.0, and 2 mM DTT. After 8 h, the dialysis solution was changed to an equal volume of buffer without urea and dialysis continued for 24 h. The final dialysis was carried out for 12 h against 0.05 M sodium phosphate, pH 8.0, and 2 mM DTT. Separation of dye-conjugated isozymes was achieved by ion-exchange chromatography. Samples were applied to a column of DEAE-Sephadex and eluted batchwise by the sequential addition of 0.05 M sodium phosphate, pH 8.0, and 2 mM DTT, then the same buffer plus 0.15 M KCl, and lastly buffer with 0.4 M KCl. Fractions containing each of the three isozymes were concentrated by using an Amicon ultrafiltration cell equipped with a YM-10 membrane.

Electrophoresis was performed by using thin films of agarose and rods of polyacrylamide gel. The agarose procedure employed instrumentation and strips supplied by Corning. Electrophoresis in 7% polyacrylamide gels was carried out according to the procedure of Davis (1964). After electrophoresis, dye-conjugated proteins were visualized under ultraviolet light (366 nm). Activity was detected with the enzyme-linked, NADH-generating CK fluorogenic assay reagent available from Corning.

Absorption spectra were recorded with a Perkin-Elmer Model 552 double-beam spectrophotometer. The reference cuvette contained the appropriate buffer to serve as blank. Extinction coefficients used in calculating the degree of labeling were as follows: muscle CK, $A^{1\%}_{1cm}$ = 8.8 at 278 nm (Kuby & Noltmann, 1962); brain CK, $A^{1\%}_{1cm}$ = 8.1 at 278 nm (Keutel et al., 1972); CPM, ϵ_{387nm} = 29 700 M^{-1} cm^{-1} (Sippel, 1981); 5′-IAF, ϵ_{489nm} = 85 000 M^{-1} cm^{-1} (Vanderkooi et al., 1977).

Fluorescence spectra and polarization were obtained by using an SLM Model 8000 photon counting spectrofluorometer. Excitation and emission slit widths were 2 and 8 nm, respectively. All spectra were acquired in the corrected mode, and all spectra displayed were corrected for solvent fluorescence. Samples were continuously stirred during the 2-min spectral acquisition period and maintained at 25 °C by circulating thermostated water through the cuvette-holder block.

Polarization values were obtained by using three Glan-Thompson calcite prism polarizers formatted in a T configuration with one for the excitation beam and two for the fluorescence emission. Fluorescence emission polarized at 0° (vertical) was passed through an MC 320 monochromator to select the appropriate emission wavelength for the dye-conjugated proteins. Emission polarized at 90° (horizontal) was passed through filters of appropriate wavelength cutoff (455 nm for CPM-conjugated protein; 478 nm for acetamidofluorescein-conjugated protein). Polarization is defined as

$$P = (F_{VV} - GF_{VH})/(F_{VV} + GF_{VH}) \quad (1)$$

where F_{VV} and F_{VH} are the relative intensities of the vertical and horizontal components of the fluorescence obtained with vertically polarized excitation energy. The factor G, used to correct for instrumental imbalances, was acquired with the excitation polarizer positioned at 90°. After a second data acquisition period with the excitation polarizer at 0°, the polarization was displayed on the data processing monitor. Each measurement was made for 10 s, and polarization values represent an average of four determinations.

Evaluation of the limiting polarization (P_0) of the dye-conjugated proteins was performed at 25 °C by using sequential additions of glycerol to increase the viscosity (η) of the solvent. A plot of the reciprocal of the polarization ($1/P$) vs. T/η with T in terms of kelvin and η in terms of centipoise gives a y intercept equivalent to $1/P_0$.

Quantum yield of the energy donor (M_cB) was measured

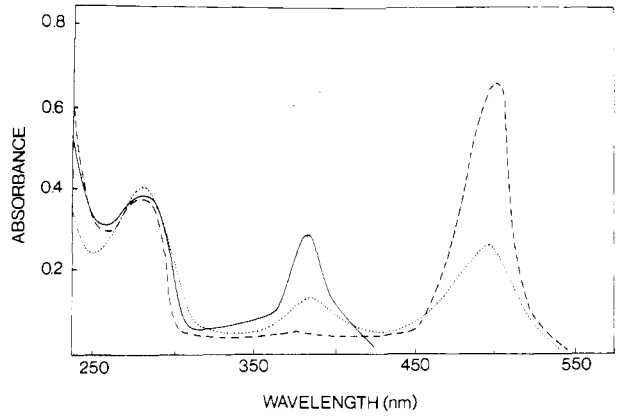

FIGURE 1: Absorption spectra of M_cM_c, B_fB_f, and M_cB_f. Samples (0.5 mL) contained 0.45 mg/mL M_cM_c (—), 0.50 mg/mL B_fB_f (---), and 0.55 mg/mL M_cB_f (···) each in 0.1 M sodium phosphate, pH 8.0, and 2 mM DTT cuvettes at 25 °C. M_cB_f was obtained after concentration of the double-labeled product shown in Figure 2. Instrumentation and additional details are given under Experimental Procedures.

by reference to the known quantum yield of quinine bisulfate (QBS) in 1 N H_2SO_4 according to the following equation:

$$\phi(M_cB) = \frac{\phi(QBS)\cdot A(QBS)\cdot \text{area}(M_cB)}{A(M_cB)\cdot \text{area}(QBS)} \qquad (2)$$

where A represents the absorbance at 350 nm and area refers to the area under the corrected emission spectrum of sample excited at 350 nm. A value of ϕ(QBS) = 0.54 (Adams et al., 1977) was used in the calculation.

Results

Analysis of M_cM_c and B_fB_f. Both native isozymes MM and BB reacted with 1.9 mol of DTNB/mol of protein. From the spectra illustrated in Figure 1 and the extinction coefficient values given under Experimental Procedures, we compute that 1.9 mol of 5′-IAF reacted per mol of isozyme BB and 1.8 mol of CPM reacted per mol of isozyme MM. Subsequent to dialysis for removal of DTT, both dye-conjugated proteins failed to react with DTNB (<0.1 mol of 5-thio-2-nitrobenzoic acid released per mol of CK protein). Following reaction of MM and BB with potassium tetrathionate, the resulting *S*-sulfenylsulfonate-derivatized proteins failed to react with either 5′-IAF or CPM (<0.2 mol of dye/mol of CK protein). B_fB_f protein was devoid of enzymatic activity, whereas M_cM_c retained approximately 11% of the activity of an unlabeled sample of equivalent concentration.

Isolation of the Products of Hybridization. The separation of the products of the hybridization of M_cM_c and B_fB_f by ion-exchange chromatography is illustrated in Figure 2. No overlap of products was observed; however, it is apparent that some B_f subunit containing material failed to adhere to the column, eluting with M_cM_c. Further analysis of the products by acrylamide gel electrophoresis showed single fluorescent bands for M_cM_c, M_cB_f, and B_fB_f, which corresponded in electrophoretic mobility to isozymes MM, MB, and BB which had been stained for activity. The absorption spectrum of isolated M_cB_f is illustrated in Figure 1. M_cB and MB_f, isolated by a similar procedure, exhibited 55% and 42% the activity of an equivalent concentration of MB, in contrast to only 4% for M_cB_f. Equal concentrations of M_cB_f, M_cB, MB_f, and MB, based on the protein assay of Bradford (1976), did not exhibit significant differences in the spectrum of the intrinsic fluorescence with respect of intensity or emission maximum (340 ± 1 nm).

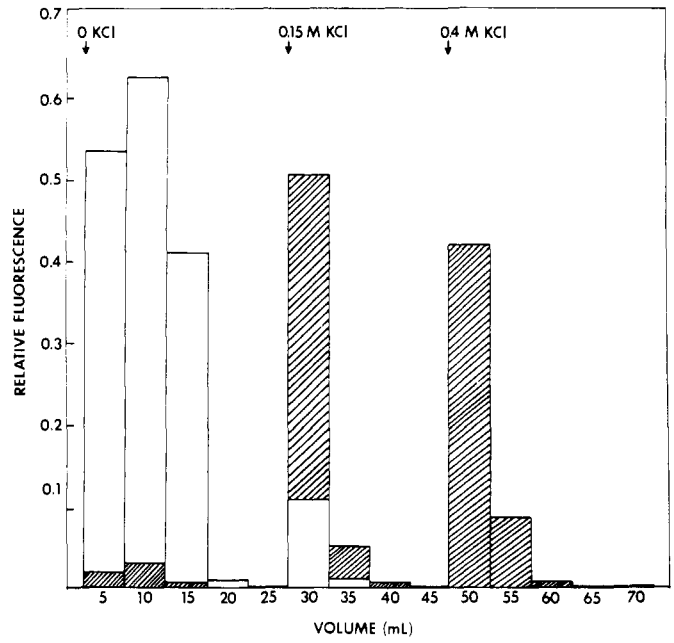

FIGURE 2: Separation of M_cM_c, M_cB_f, and B_fB_f by ion-exchange chromatography. Batchwise elution pattern of the products of the hybridization of M_cM_c and B_fB_f. Sample containing 2.7 mg of total protein in 4.0 mL of 0.02 M sodium phosphate, pH 8.0, and 2 mM DTT was applied to a column (1.5 × 5 cm) of DEAE-Sephadex equilibrated with the same buffer. The column was developed by sequential elution with buffer, buffer plus 0.15 M KCl, and buffer plus 0.4 M KCl. Fractions (5 mL) were analyzed for fluorescence at excitation = 387 nm and emission = 470 nm (open bars) and excitation = 490 nm and emission = 510 nm (hatched bars).

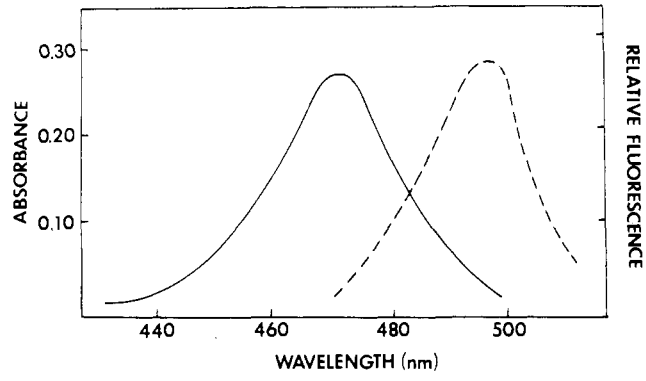

FIGURE 3: Overlap spectra. Fluorescence emission spectrum of M_cB (—) and absorption spectrum of MB_f (---). Excitation wavelength 387 nm. Each sample (0.52 mg/mL MB_f; 0.15 mg/mL M_cB) was in 0.1 M sodium phosphate, pH 8.0, and 2 mM DTT.

Evaluation of J and Q. The spectral overlap integral (J) between the donor emission and the acceptor absorption was computed from the following equation:

$$J = \frac{\int \epsilon_\lambda(d\lambda)F_\lambda(d\lambda)\lambda^4}{\int F_\lambda(d\lambda)} \qquad (3)$$

where λ is the wavelength, F_λ is the relative fluorescence of the donor as a function of λ, and ϵ_λ is the molar extinction coefficient of the acceptor as a function of λ (Förster, 1965). The fluorescence emission of M_cB and the absorption of MB_f displayed spectral overlap between 470 and 498 nm (Figure 3). The extinction coefficient of MB_f was computed at each integer value within the wavelength range. Similar evaluation of the relative fluorescence at each integer value in the wavelength range of spectral overlap was determined for the donor, M_cB. Substitution of these values into the summation

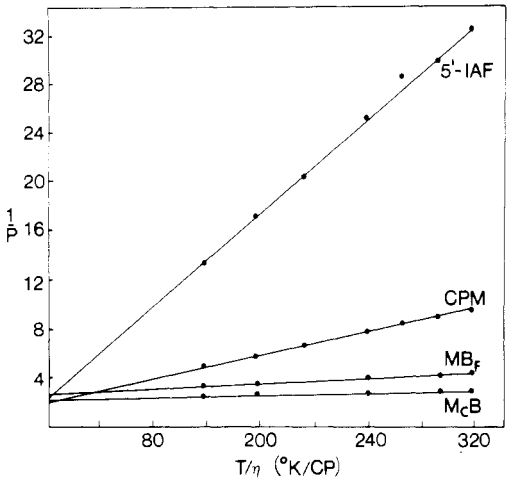

FIGURE 4: Polarization of dyes and dye-conjugated proteins. Samples analyzed consisted of 5′-IAF (1 μg/mL), CPM (1 μg/mL), MB_f (0.6 μM), or M_fB (0.5 μM) in 0.1 M sodium phosphate, pH 8.0, and 2 mM DTT. Viscosity (η), evaluated from the table listed by Dean (1973), was varied isothermally (25 °C) by the addition of glycerol. Values of the limiting polarization (P_0) were obtained by extrapolating to infinite viscosity. Each point is the average of four measurements, and for a given sample, P did not vary by more than ±0.004. Additional details are given in the text.

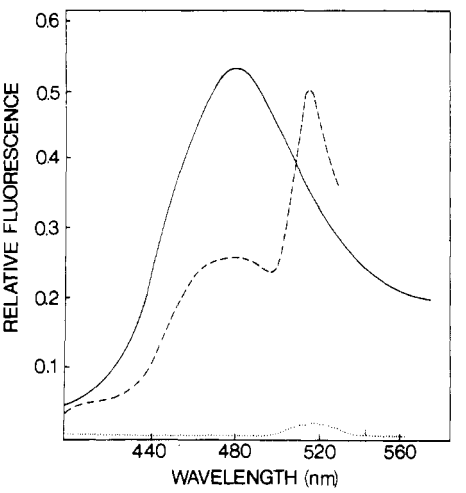

FIGURE 5: Corrected emission spectra of M_cB (—), M_cB_f (- - -), and MB_f (···). Samples contained 1.2 μM protein in 0.1 M sodium phosphate, pH 8.0, and 2 mM DTT. Fluorescence excitation wavelength was 387 nm. (Not shown) The sample of MB_f excited at 490 nm exhibited a relative fluorescence of 2.15 and emission λ_{max} at 515 nm.

equation (eq 3) yields a result of $J = 1.56 \times 10^{-13}$ cm^3 M^{-1}.

The quantum yield (ϕ) of the energy donor M_c was measured as described under Experimental Procedures and eq 2. The value obtained was 0.14.

Fluorescence polarization analyses and estimation of K^2: Evaluation of R_0 requires a knowledge of K^2, the dipole–dipole orientation factor (eq 4, below). This factor, which relates the orientation of the donor and acceptor transition dipoles with respect to each other, is often unknown. The theoretical range is 0–4 (Dale et al., 1979). For the case where the donor and acceptor ligands are free to rotate at a rate faster than the excited-state lifetime of the donor, an average value of $K^2 = 0.66$ may be used. When ligands are fixed, and the orientation uniquely defined but unknown, a value of $K^2 = 0.475$ is often used, which represents an average value for a collection of fixed but random orientations. If neither of these situations obtains, it is necessary to determine the magnitude of the depolarization factors as described by Dale et al. (1979).

Evaluation of the polarization of the donor and acceptor protein-bound ligands, when compared with the limiting polarization (P_0) of the free ligands (conditions under which the ligand is confined to a rigid medium), gives a qualitative estimate of the rotational mobility of the ligand when conjugated to the protein. Figure 4 illustrates the results of the determination of the P_0 values for CPM, 5′-IAF, M_cB, and MB_f. A comparison of the P_0 for CPM with the P value for M_cB at 25 °C in buffer, 0.422 and 0.416, respectively, indicates that the dye, when coupled to the protein, is essentially immobilized. A similar comparison of P_0 for 5′-IAF (0.400) and P for MB_f at 25 °C in buffer (0.360) suggests some limited mobility; however, the value of $P = 0.036$ for 5′-IAF at 25 °C in buffer indicates that when covalently attached to the B subunit of MB, acetamidofluorescein rotation is severely restricted. From these observations we conclude that the ligands are arranged in a nearly immobile and unique orientation. An estimate of the range of K^2 was determined as described by Dale et al. (1979) after evaluation of various anisotropy (r) values and the depolarization factors. The ratios r_{0D}/r_{fD} and r_{0A}/r_{fA} are an estimate of the degree of rotational freedom of the donor and acceptor, where r_{0A} and r_{0D} are the measured limiting anisotropies of the donor and acceptor bound to protein, respectively, and r_{fD} and r_{fA} are the fundamental anisotropies of the free fluorophores. According to the notation of Dale et al. (1979) these ratios are designed $\langle d_D \rangle$ and $\langle d_A \rangle$, the observed depolarization factors, and are the numerical square roots of the axial depolarization factors, $\langle d_D^x \rangle$ and $\langle d_A^x \rangle$. For M_cB and MB_f, $\langle d_D^x \rangle$ and $\langle d_A^x \rangle$ are 0.982 and 0.910, respectively. These values can then be used to determine the axial transfer depolarization factor $\langle d_T^x \rangle$ according to

$$\langle d_T \rangle = \langle d_D^x \rangle \langle d_T^x \rangle \langle d_A^x \rangle \quad (4)$$

where $\langle d_T \rangle$ is the average transfer depolarization. Evaluation of $\langle d_T \rangle$ is derived from $\langle d_T \rangle = r_{DA}/0.4$, where r_{DA} is the transfer anisotropy, experimentally determined by measuring the anisotropy of the donor–acceptor pair, when exciting into the lowest energy band of the donor (422 nm for M_c) and selecting for the emission of the sensitized acceptor (515 nm for B_f). The axial transfer depolarization factor for M_cB_f was 0.6. The parameters $\langle d_D^x \rangle$, $\langle d_A^x \rangle$, and $\langle d_T^x \rangle$ were then used in combination with the appropriate contour plot (Figure 6, Dale et al., 1979) for estimation of the range of K^2, resulting in values from 0.06 to 3.3. Consideration of the effect of errors in K^2 is described under Discussion.

Calculation of R_0. The distance at which energy transfer is 50% efficient (R_0) is, according to Förster (1965)

$$R_0 = (JK^2\phi_0 n^{-4})^{1/3}(9179 \times 10^3) \quad (5)$$

Substituting the values for J, K^2, and ϕ_0 and assuming a value for the refractive index of $n = 1.33$, we derive the value R_0 = 29–53 Å.

Calculation of E. The efficiency of energy transfer, E, was determined by measuring the extent of donor quenching (λ_{emit} 468 nm) in the presence of acceptor according to

$$E = 1 - F^{\lambda=468}_{(M_cB_f)}/F^{\lambda=468}_{(M_cB)} \quad (6)$$

and by the relative increase in acceptor fluorescence described by (Schiller, 1975)

$$E = [A(M_cB)/A(MB_f)][F^{\lambda=515}_{(M_cB_f)}/F^{\lambda=515}_{(MB_f)}] \quad (7)$$

where A = absorbance at the absorption maximum of the donor and F the fluorescence of the acceptor (λ_{emit} 515 nm) resulting from excitation at the absorption maximum of the donor. Data used to obtain $F(M_cB_f)/F(M_cB)$ at λ 468 nm was acquired from the spectral data illustrated in Figure 5.

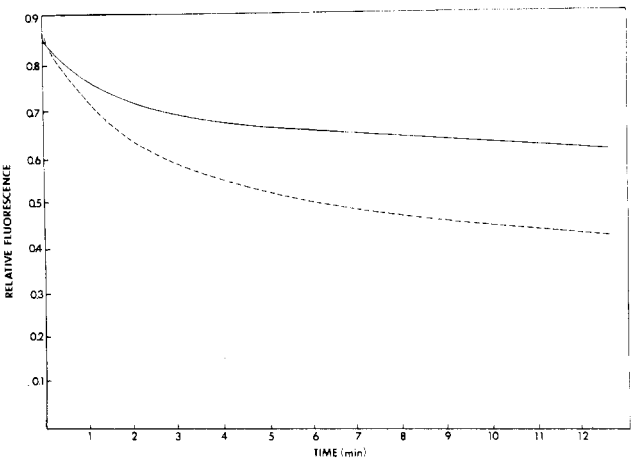

FIGURE 6: Kinetics of hybridization. Samples of dye-conjugated proteins were incubated for 1 h in 8 M urea in 0.1 M sodium phosphate, pH 8.0, and 2 mM DTT. Aliquots (0.02 mL) consisting of a mixture of denaturated M_cM_c and B_fB_f (- - -) or M_cM_c and BB (—) were added to 2.0 mL of buffer in a continuously stirred, thermostated fluorescence cuvette to a final concentration of 24 μg/mL for each protein. The sample addition and initial mixing interval was 4 s. Excitation and emission wavelengths were 387 and 470 nm, respectively.

Sensitized fluorescence $F^{\lambda=515}_{(M_cB_f)}$ and direct fluorescence $F^{\lambda=515}_{(M_cB_f)}$ were also acquired from the data in Figure 5. The energy-transfer efficiency was 0.56 and 0.54 as determined from the donor quenching and the sensitized acceptor fluorescence, respectively.

Evaluation of R. The distance, R, between the donor and the acceptor was calculated from the equation

$$R = R_0(1/E - 1)^{1/6} \quad (8)$$

By use of the range of values for R_0 of 29 and 53 Å and E of 0.54–0.56, the solution of eq 8 yields R from 27 to 52 Å as the estimated range of distance between the sulfhydryl-labeled active sites of isozyme MB.

Kinetics of Reassembly. The kinetics of reassembly of denatured M_cM_c and B_fB_f was monitored by measuring the decrease in fluorescence emission at 468 nm. As illustrated in Figure 6, reassembly of dye-conjugated MM with B_fB_f or with unlabeled BB exhibits a pronounced initial decrease in fluorescence that is adequately described by a first-order rate constant of $k_1 = 3.2 \times 10^{-3} \pm 0.6\ s^{-1}$. The second kinetic phase is characterized by a more protracted decrease in fluorescence but is of considerably greater magnitude when acetamidofluorescein-conjugated BB rather than unlabeled isozyme is present during association. The time courses of the fluorescence when normalized to percent quenching are nearly coincidental for the reassembly in the presence and absence of the acceptor ligand.

Discussion

The Förster critical transfer distance depends upon the orientation factor, K^2, which is a function of the relative orientations of the donor and acceptor. It is clear that we have no information about the orientation except that the donor and acceptor are relatively fixed and uniquely oriented. The usual assumptions of isotropic distribution of the orientation of the donor and acceptor ($K^2 = 0.66$) or the presence of an ensemble of fixed by random orientations ($K^2 = 0.475$) are unfounded. From determination of the various anisotropy values, we have computed the observed and axial depolarization factors as well as the transfer depolarization factor. Using the contour plot developed by Dale et al. (1979), we have determined that K^2 can vary from 0.06 to 3.3. This results in a range of values for R_0 of 29–53 Å. If the theoretical value for the fundamental anisotropy of 0.4 is used in calculating the depolarization factors rather than our experimentally determined values, the range of R_0 is narrowed only from 30 to 52 Å. From our evaluation of the efficiency of energy transfer between the fluorescent donor and acceptor at the active sites of CK-MB (54–56%), we derive that the distance between the active sites may range from 27 to 52 Å. The significance of this range with respect to an assessment of the arrangement of the subunits in the hybrid isozyme is considered below.

The two fluorophores used in the present study are well suited for a measurement of energy transfer between the active sites of creatine kinase. Both react specifically with sulfhydryl groups. Creatine kinase contains a highly reactive cysteine residue at the active site of each subunit (Mahowald et al. 1962). A second feature is the considerable overlap between the fluorescence emission of CPM and the absorption of 5′-IAF. The absence of absorption of 5′-IAF at the excitation maximum of CPM precludes quenching of the excitation energy (at 378 nm) by 5′-IAF.

The specificity of the labeling of the sulfhydryl groups was demonstrated by the combined observations that approximately two dye molecules were bound per molecule of dimer and that subsequent to dye conjugation, the protein failed to react with DTNB. Moreover, *S*-sulfenylsulfonate-derivatized unlabeled protein prepared with potassium tetrathionate failed to react with either dye, suggesting that other reactive residues, including lysine, were not modified during conjugation of the native enzymes with the fluorescent ligands. It is unlikely that other sulfhydryl groups reacted, since exposure of the less reactive cysteine residues require denaturing conditions, e.g., at least 2 M urea (Grossman et al., 1981). The less than stoichiometric binding may be a reflection of a small overestimation of the absorptivity or incomplete reaction. If the values obtained do represent less than stoichiometric reaction with the cysteines, the effect of 6% more labeling of B subunit with acetamidofluorescein than M subunit with CPM in the M_CB_f hybrid is to reduce the transfer efficiency by approximately 3%.

The distinct isoelectric points (Yue et al., 1968; Grossman & Mollo, 1979) and ion-exchange behavior of the three isozymes of creatine kinase allowed for facile separation. Isolabeled M_cM_c and B_fB_f were readily separated from heterolabeled M_cB_f by chromatography with DEAE-Sephadex. This procedure is used clinically to isolate isozyme MB as part of the diagnosis of myocardial infarction (Mercer, 1974). The separation is complete if the eluting buffers are maintained at the proper ionic strengths.

Native isozyme MB and unlabeled isozyme prepared by hybridization exhibit identical electrophoretic mobilities and appear to be the same protein. That the electrophoretic mobility of the dye-conjugated M_cB_f and unlabeled MB is indistinguishable suggests that major conformational changes have not been produced by labeling of the isozyme. This is supported by the observation that both M_cB and MB_f exhibit enzymatic activity. Furthermore, we did not observe a significant perturbation in the spectral characteristics of the intrinsic fluorescence of dye-conjugated products when compared with the spectrum of native MB.

That the quenching of M_c fluorescence by association with a B_f subunit is due to the energy transfer is supported by the appearance of an emission peak with a maximum fluorescence at 515 nm. This corresponds to the emission of the acetamidofluorescein conjugate to the B subunit. It cannot be attributed to direct excitation of the dye, as demonstrated by

the negligible fluorescence of MB_f excited at 387 nm, but reasonably reflects excitation of the dye due to emission of M_c when dimerized with B_f in M_cB_f. The transfer efficiency determined from the magnitude of the sensitized emission of the acceptor is in close agreement with that determined by donor quenching. Furthermore, an excitation spectrum of M_cB_f at the maximum emission wavelength of acetamidofluorescein (515 nm) reveals an excitation peak at 390 nm characteristic of the presence of CPM.

Isozyme MM is the most thoroughly characterized form of creatine kinase. The subunits are arranged in the dimer to give an axial ratio of 4 for an assumed anhydrous prolate ellipsoid (Yue et al., 1967). X-ray crystallographic studies (McPherson, 1973) reveal that the dimer possesses a 2-fold axis of symmetry with dimensions of $a = 47$ Å, $b = 86$ Å, and $c = 125$ Å. There is evidence that the dimers are asymmetrically arranged (Degani & Degani, 1980); however, this view is contrary to the sedimentation velocity and X-ray crystallography studies. One report (Degani & Degani, 1979) indicates that isozymes BB and MB are also composed of asymmetrically arranged subunits. The commercial preparations, reportedly studied without further purification, have proven in our hands to consist of less that 20% creatine kinase and, in the case of the preparation from heart, a mixture of isozymes. Sedimentation velocity studies (Yue et al., 1968) of purified CK-BB reveal that the conformation of this isozyme is the same as that of the muscle-type isozyme.

Energy-transfer analysis (Haugland, 1975) and cross-linking studies (Bickerstaff & Price, 1978) suggest that the active sites of creatine kinase MM are well separated. Neither study gives an estimate of the distance, but Haugland states that the active sites are likely to be separated by greater than 30 Å. Our results are in agreement with these findings. We can further suggest that if the subunits of MB are arranged as they are in the homodimers and given the dimensions of the dimer determined from sedimentation velocity and X-ray crystallographic analysis, it can be concluded that the active sites cannot be located at the ends of the subunits and must be situated closer to the central short axis of the dimer. This would be the case regardless of which value for K^2 is used for the assessment of R_0. Furthermore, it is clear that our results eliminate the possibility of direct interaction between the active sites of creatine kinase during catalysis. Therefore, site–site interaction cannot be invoked as an explanation to account for the synergism observed in substrate binding (Watts, 1973; Grossman, 1983).

Several possible descriptions of the subunit arrangement in CK-MB may be considered. If the active site of the B subunit is located in the same position on the polypeptide chain as in the M subunit, and given that the M and B subunits have similar overall conformation, energy transfer between the active sites would occur if the subunits were arranged contiguously and parallel (as opposed to antiparallel) along the longitudinal axis. A second possibility is that the active sites of the B subunit and M subunit are not located in the same place along their respective polypeptide chains, so that in the hybrid, the proximity of the active sites is within the limits for energy transfer (<60 Å). Finally, subunits may be asymmetrically arranged, either by virtue of association of essentially rigid polypeptide chains or by dimerization-induced conformational changes. Relevant to the latter possibility, isozyme BB has been shown to consist of subunits that are more "open" and more flexible than isozyme MM in response to interaction with substrate (Jacobs & Kuby, 1970; Grossman, 1983).

The demonstration that resonance energy transfer occurs between the active sites of CK-MB suggested to us that donor quenching during hybridization could be used to distinguish between subunit renaturation and subunit association. The kinetics of reassembly were monitored by the decrease in donor fluorescence upon association with the acceptor. During this procedure, the formation of the hybrid is accompanied by regeneration of the parent homodimers, and changes in fluorescence contain components due to reassembly of all three dimers. The hybridization between M_cM_c and BB displays a rapid initial decrease in fluorescence, followed by a more protracted phase with minor change in fluorescence. These changes can be attributed to formation of M_cM_c and M_cB from denatured M_c and B subunit. The decline in fluorescence intensity cannot be due to energy transfer because of the large Stokes shift of CPM or to energy transfer, quenching, or inner filter effects due to intrinsic fluorophores. It likely reflects changes in the conformation of the polypeptide chain during refolding. The kinetics of the first phase ($k_1 = 3.2 \times 10^{-3} \pm 0.6$ s^{-1}) are very similar to the kinetics of the refolding of urea-denatured CK-MM (Grossman et al., 1981) monitored by changes in intrinsic fluorescence ($k_1 = 4.7 \times 10^{-3} \pm 0.7$ s^{-1}). When dye-conjugated B subunit is present with M_c during renaturation, the initial rapid decline in donor fluorescence also occurs, and a subsequent decline is then evident which is of considerably greater magnitude than when unlabeled B subunit is used. This decrease in the second kinetic phase may be attributed to energy transfer accompanying a reshuffling of subunits to form the native dimer or the association of native subunits. The finding that the percent change in fluorescence of donor as a function of reassembly time is independent of the presence of the acceptor is consistent with a mechanism involving slow refolding of subunits followed by rapid subunit association. This interpretation is also consistent with our previous kinetic studies (Grossman et al., 1981) and an analysis of the reassembly of cross-linked CK (Grossman et al., 1983) demonstrating that subunit association occurs after subunit renaturation.

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References

Adams, M. J., Highfield, J. G., & Kirkbright, G. F. (1977) *Anal. Chem. 49*, 1850–1852.

Bickerstaff, G. F., & Price, N. C. (1978) *Int. J. Biochem. 9*, 1–8.

Böhlen, P., Stein, S., Dairman, W., & Udenfriend, S. (1973) *Arch. Biochem. Biophys. 155*, 213–220.

Bradford, M. (1976) *Anal. Biochem. 72*, 248–254.

Bulcke, J. A., & Sherwin, A. L. (1969) *Immunochemistry 6*, 681–687.

Dale, R. E., Eisinger, J., & Blumberg, W. E. (1979) *Biophys. J. 26*, 161–194.

Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404–427.

Dean, J. A. (Ed) (1973) *Lange's Handbook of Chemistry*, 11th ed., pp 10–288, McGraw-Hill, New York.

Degani, C., & Degani, Y. (1980) *J. Biol. Chem. 255*, 8211–8228.

Degani, Y., & Degani, C. (1979) *Biochemistry 18*, 5917–5923.

Eppenberger, H. M., Dawson, D. M., & Kaplan, N. O. (1967) *J. Biol. Chem. 242*, 204–209.

Förster, T. (1965) *Mod. Quantum Chem., Lect. Istanbul Int. Summer Sch., 1964, Sect. 111-B*, 93–137.
Grossman, S. H. (1983) *J. Neurochem. 41*, 729–736.
Grossman, S. H., & Mollo, E. (1979) *Int. J. Biochem. 10*, 367–381.
Grossman, S. H., Pyle, J., & Steiner, R. J. (1981) *Biochemistry 20*, 6122–6128.
Grossman, S. H., LeJeune, B., & Mixon, D. (1983) *Arch. Biochem. Biophys. 224*, 449–455.
Habeeb, A. F. S. A. (1972) *Methods Enzymol. 25*, 457–464.
Haugland, R. P. (1975) *J. Supramol. Struct. 3*, 192–199.
Jacobs, H. K., & Kuby, S. A. (1970) *J. Biol. Chem. 245*, 3305–3314.
Keutel, H. J., Okabe, K., Jacobs, H. K. Ziter, F., Maland, L., & Kuby, S. A. (1972) *Arch. Biochem. Biophys. 150*, 648–678.
Kuby, S. A., & Noltmann, E. A. (1962) *Enzymes, 2nd Ed. 6*, 514–596.
Mahowald, T. A., Noltmann, E. A., & Kuby, S. A. (1962) *J. Biol. Chem. 237*, 1535–1548.
McPherson, A., Jr. (1973) *J. Mol. Biol. 81*, 83–86.
Mercer, D. W. (1974) *Clin. Chem. 20*, 36–40.
Morel-Deletraz, F. (1979) *Enzyme 27*, 277–279.
Morrison, J. F., & James, E. (1965) *Biochem. J. 97*, 37–52.
Rosalki, S. B. (1967) *J. Lab. Clin. Med. 69*, 696–705.
Schiller, P. W. (1975) in *Biochemical Fluorescence: Concepts* (Chen, R. F., & Edelhoch, H., Eds.) pp 285–303, Marcel Dekker, New York.
Sippel, T. O. (1981) *J. Histochem. Cytochem. 29*, 1377–1381.
Stryer, L. (1978) *Annu. Rev. Biochem. 47*, 819–846.
Trudinger, P. A. (1961) *Biochem. J. 78*, 680–686.
Vanderkooi, J. M., Ierokomas, A., Nakamura, H., & Martonosi, A. (1977) *Biochemistry 16*, 1262–1267.
Watts, D. C. (1973) *Enzymes, 3rd Ed. 8*, 383–455.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.
Yue, R. H., Palmieri, R. H., Olson, O. E., & Kuby, S. A. (1967) *Biochemistry 6*, 3204–3227.
Yue, R. H., Jacobs, H. K., Okabe, K., Keutel, H. J., & Kuby, S. A. (1968) *Biochemistry 7*, 4291–4298.

Purification and Characterization of Six Cytochrome P-450 Isozymes from Human Liver Microsomes†

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ABSTRACT: Six cytochrome P-450 (P-450) isozymes were purified to electrophoretic homogeneity from the livers of four human organ donors, with three of these isozymes purified from a single individual. Differences were noted between all six P-450s for some or all of the parameters determined by the techniques of sodium dodecyl sulfate–polyacrylamide gel electrophoresis, peptide mapping, spectral analysis of ferrous–carbon monoxide complexes, double-diffusion immunoprecipitin analysis or crossed immunoelectrophoresis (sodium dodecyl sulfate–polyacrylamide gel electrophoresis/peroxidase-coupled staining) with rabbit antisera raised to five of the P-450s, or catalytic activity toward *d*-benzphetamine, benzo[*a*]pyrene, acetanilide, debrisoquine, (*R*)- and (*S*)-warfarin, and 1-naphthylamine. While NADPH-fortified human liver microsomal preparations showed catalytic activity toward trichloroethylene, 7-ethoxycoumarin, 2-naphthylamine, and 2-aminofluorene in addition to the other substrates mentioned, none of the P-450s which we purified from these microsomes catalyzed the oxidation of these compounds in reconstituted enzyme systems containing purified rat liver NADPH–P-450 reductase. Antibodies raised against one of the purified P-450s inhibited *d*-benzphetamine *N*-demethylase activity in microsomal incubations but did not inhibit the metabolism of 7-ethoxycoumarin, acetanilide, benzo[*a*]pyrene, or debrisoquine. The data provide a strong biochemical basis for the view that distinct isozymes of P-450 exist in humans and that these isozymes differ in catalytic activity toward drugs and carcinogens.

Interindividual variations in the metabolism of foreign compounds such as therapeutic drugs have been known for many years. Both beneficial and harmful effects to patients can result from unusually slow or rapid metabolism, depending upon the situation under consideration (Conney, 1982; Idle & Smith, 1979; Penno et al., 1981; Ritchie et al., 1980; Sjöqvist & von Bahr, 1973). Such variation appears to have a strong genetic component (Dayer et al., 1982; Nebert & Felton, 1976; Penno et al., 1981; Sloan et al., 1981; Vesell, 1977), although environmental modifications of metabolizing capability (Alvares et al., 1979; Conney, 1982; Nebert et al., 1969) and intraindividual tissue variation (Autrup et al., 1982) must also be taken into consideration. The variation in metabolism is exemplified by the case of debrisoquine, where approximately 10% of the population metabolizes the drug relatively slowly (Kahn et al., 1982; Mahgoub et al., 1977; Ritchie et al., 1980; Sloan et al., 1981). A similar fraction of the human population metabolizes a number of other drugs at unusually low rates, including phenformin, guanoxan, phenacetin, sparteine, metaprolol, bufaralol, perhexiline, mucodyne, and mephenytoin (Kahn et al., 1982, and references therein). Variations in

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