Characterization of the Internal Calcium(II) Binding Sites in Dissolved Insulin Hexamer Using Europium(III) Fluorescence†

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ABSTRACT: The fluorescence of Eu(III) is used to study the nature of the Ca(II) binding sites in the central cavity of the two-zinc(II) insulin hexamer. The dependence of the Eu(III) fluorescence lifetime upon Eu(III) stoichiometry indicates that there are three identical Eu(III) binding sites present in the two-zinc(II) insulin hexamer in solution. Addition of excess Ca(II) causes a decrease in the Eu(III) fluorescence intensity, confirming that Ca(II) competes for the observed Eu(III) sites. The solvent dependence of the Eu(III) fluorescence lifetime (H_2O vs. D_2O) indicates that four OH groups are coordinated to each Eu(III) in the hexamer. Substitution of Co(II) for Zn(II) causes a decrease in the Eu(III) fluorescence lifetime. Calculations based on Förster energy-transfer theory predict that the Co(II) [or Zn(II) in vivo] and Eu(III) [or Ca(II) in vivo] binding sites are separated by 9.6 ± 0.5 Å. Variation of the metal stoichiometries indicates that all three Eu(III) [or Ca(II) in vivo] sites are equidistant from the Zn(II) sites. We conclude that these sites are identical with the three central Zn(II) sites present in insulin hexamer crystals soaked in excess Zn(II) [Emdin, S. O., Dodson, G., Cutfield, J. M., & Cutfield, S. M. (1980) *Diabetologia 19*, 174–182] and suggest that these central sites are occupied by Ca(II) in vivo.

Insulin is a small polypeptide hormone (~5700 daltons) that plays a major role in the regulation of mammalian carbohydrate, amino acid, and fat metabolism (Czech, 1977). It is synthesized in the pancreatic β-cell and stored there in the form of crystalline hexamer containing Zn(II) (Pihl, 1968; Greider et al., 1969; Howell et al., 1969; Figlewicz et al., 1980). Insulin recrystallized in vitro contains two Zn(II) atoms per hexamer (Schlichtkrull, 1956; Brill & Venable, 1968). X-ray crystallographic studies show that the two-zinc(II) insulin hexamer is torus shaped (~50 Å in diameter by 35-Å high, see Figure 1), with each Zn(II) ion located 8.5 Å from the hexamer center along the 3-fold symmetry axis traversing the central cavity (Blundell et al., 1972).

In addition to Zn(II), Ca(II) is present in high concentration in pancreatic storage granules (Herman et al., 1973; Schäfer & Klöppel, 1974; Howell et al., 1975; Ravazzola et al., 1976). It has previously been suggested that Ca(II) may have a role in insulin storage (Hellman et al., 1976; Howell et al., 1978; Andersson & Berggren, 1979) and may be involved in the formation of crystalline Zn(II) insulin (Kohnert et al., 1979). Recent studies indicate that insulin secretion in response to glucose is mediated by Ca(II) (Malaisse et al., 1975; Hellman, 1977; Wollheim et al., 1978). It appears that the granules form a nonlabile pool of Ca(II) that is responsible for the long-term release of insulin (Klöppel & Bommer, 1979; Andersson & Berggren, 1979; Berggren, 1980), suggesting that there are specific Ca(II) binding sites in the insulin hexamer. The presence of Ca(II) binding sites on the surface of the crystalline hexamer (Pitts et al., 1980) and in the central cavity of the hexamer in solution (Sudmeier et al., 1981) has been reported recently. The external sites are involved in cross-linking three hexamers together and are believed to aid in the formation of crystalline Zn(II) insulin hexamer at pH 7.0. The internal site is not accessible to the solvent and may be formed by six B-13 glutamate residues present in the center of the hexamer. Three equivalent Zn(II) binding sites in the hexamer center between two B-13 glutamate residues paired by the association of insulin dimers in hexamer crystals soaked in excess Zn(II) have recently been reported (Emdin et al., 1980). Because oxygen ligands generally prefer Ca(II) over Zn(II), we suspected that in the presence of high Ca(II) concentrations, the three central sites may be occupied by Ca(II). In order to test this hypothesis, we undertook these studies of laser-induced fluorescence of insulin-bound europium(III).

Although trivalent lanthanide ions such as Eu(III) do not occur naturally in biological systems, they are useful probes for Ca(II) binding sites in macromolecules (Nieboer, 1975; Reuben, 1975). Replacement of Ca(II) with lanthanide ions is isomorphous and generally maintains biological activity (Mathews & Weaver, 1974; Moews & Kretsinger, 1975; Reuben, 1977). Laser-induced lanthanide fluorescence is a useful probe for determining distances between appropriate pairs of metal ion binding sites in macromolecules (Horrocks et al., 1975) and metal hydration numbers (Horrocks & Sudnick, 1979).

The number of water molecules coordinated to Eu(III) bound in the Ca(II) binding sites of the Zn(II) insulin hexamer can be determined by observation of fluorescence lifetimes of Eu(III) hexamer samples prepared in H_2O and in D_2O. The experimental fluorescence decay constant k_{obsd} of Eu(III) in H_2O can be written as

$$k_{obsd} = k_R + k_X + k_{H_2O}\chi_{H_2O} \quad (1)$$

where k_R is the natural radiative decay constant, k_X is the nonradiative decay constant excluding relaxation via OH vibrations, and k_{H_2O} is the decay rate due to the numbers of waters, χ_{H_2O}, in the first coordination sphere. When the sample is prepared in D_2O, the observed rate of decay is described by the following equation:

$$k_{obsd} = k_R + k_X + k_{D_2O}\chi_{D_2O} \approx k_R + k_X \quad (2)$$

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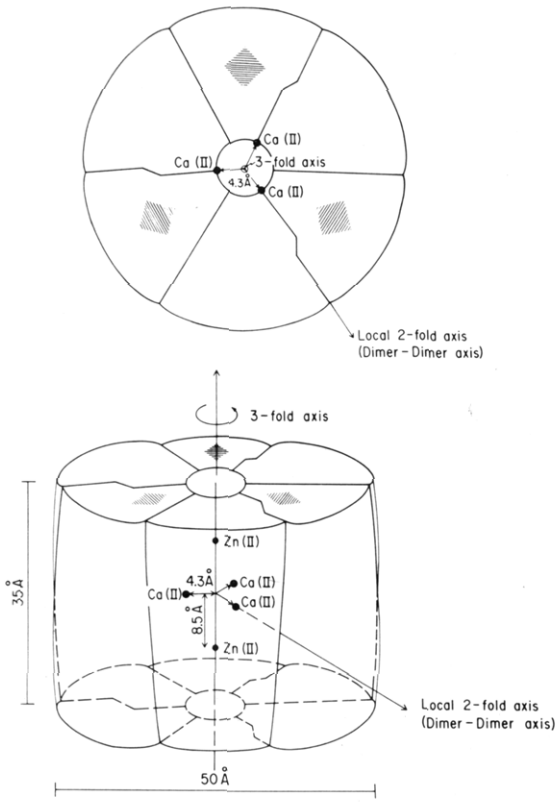

FIGURE 1: Three-dimensional structural representation of the two-zinc(II) insulin hexamer based on the X-ray crystal structure (see text). The jagged lines between monomers on the top and bottom of hexamer indicate a dimer–dimer interface. The straight lines between monomers on the top and bottom of the hexamer indicate a monomer–monomer interface within a dimer. The "hash marks" indicate the top of monomers. The proposed Ca(II) binding sites are shown in the center of the hexamer. The X-ray crystal structure was obtained from a Zn-saturated sample in which the three central sites were occupied by Zn(II) (see text).

Eu(III) fluorescence from the $^5D_0$ level to the ^7F_J manifold is the predominant radiative mode. However, vibrational transitions inhomogeneously broadened by the presence of H_2O or D_2O in the first coordination sphere create a band of energies through which the vibrational levels of OH and OD can couple (Haas & Stein, 1971).

The difference in energy of the various vibrationally excited states of H_2O and D_2O accounts for the change in the observed rates of relaxation of Eu(III). Figure 2 shows the energy-level diagram for Eu(III) and the energies of the various vibrational overtones of the OH and OD oscillators. It can be seen that relaxation via OH oscillators requires the creation of the fourth vibrational overtone (Heller, 1966). The ratio of the probabilities for creation of an OH mode in the $v = 4$ vibrationally excited state to that of OD in the $v = 5$ excited has been calculated to be greater than 250:1 (Haas & Stein, 1972); hence, k_{D_2O} is a negligible term in eq 2.

Subtraction of eq 2 from eq 1 results in a quantity that can be related to the number of water molecules coordinated to Eu(III):

$$\Delta k_{obsd} = k_{H_2O}\chi_{H_2O} \quad (3)$$

A systematic study of crystalline and solution metal complexes of known coordination has been presented (Horrocks & Sudnick, 1979). A linear least-squares fit to these data yields a k_{H_2O} value of 0.974 ms^{-1}. Horrocks and Sudnick applied this technique to two proteins with well-characterized Ca(II) binding sites, parvalbumin and thermolysin. Their results were consistent with the known numbers of coordinated water

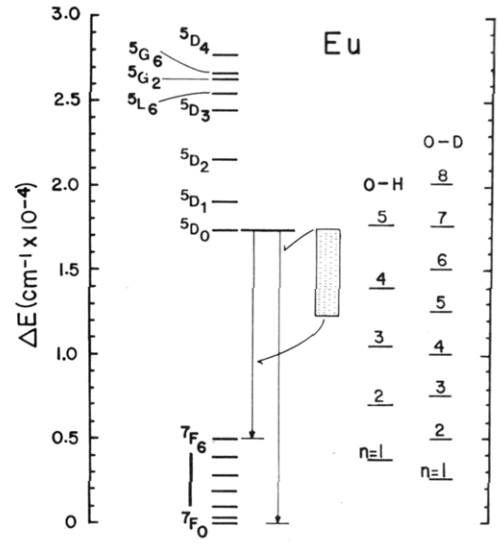

FIGURE 2: Eu(III) fluorescence from the $^5D_0$ level to the ^7F_J manifold can couple to vibrational overtones of OH and OD by vibrational transitions broadened by collisions with H_2O or D_2O. This broadening, on the order of 100 cm^{-1}, creates a band of energies (shown as the shaded region) between 1.2×10^4 and 1.7×10^4 cm^{-1} that overlap with the fourth and fifth vibrational levels of the OH and OD stretching modes, respectively. The difference in probabilities for creating the fourth and fifth vibrational levels of the OH or OD stretching modes accounts for the difference in the rates of the nonradiative relaxation via OH and OD stretching vibrations.

molecules. Our investigation of insulin is an application of this method to a relatively uncharacterized Ca(II) binding site. Similar approaches have been used to study troponin C (Wang et al., 1981) and human prothrombin (Rhee et al., 1982).

The distance of the Ca(II) binding site from the two Zn(II) sites was determined by the observation of the Eu(III) fluorescence quenching upon substitution of Co(II) for Zn(II). The observed fluorescence quenching ratio I/I_0 is related to the distance R between Co(II) and Eu(III) (Förster, 1965):

$$I/I_0 = 1/[1 + (R_0/R)^6] \quad (4)$$

where I and I_0 are the fluorescence intensities with and without Co(II) and R_0 is the critical radius at which 50% of the energy is transferred to the quencher. The critical radius is calculated from the following expression:

$$R_0^6 = (8.78 \times 10^{-25})\kappa^2 Q n^{-4} J \text{ cm}^6 \quad (5)$$

where Q is the fluorophore quantum yield in the absence of energy transfer, κ is the dipolar orientation factor, n is the index of refraction of the surrounding medium, and J is the spectral overlap integral given by

$$J = \frac{\int_0^\infty F(\tilde{\nu})\epsilon(\tilde{\nu})\tilde{\nu}^{-4}\, d\tilde{\nu}}{\int_0^\infty F(\tilde{\nu})\, d\tilde{\nu}} \quad (6)$$

where $\epsilon(\tilde{\nu})$ is the molar extinction coefficient of the acceptor in units of cm^{-1} M^{-1}, $\tilde{\nu}$ is the frequency in wavenumbers (cm^{-1}), and $F(\tilde{\nu})$ is the fluorescence distribution function.

EXPERIMENTAL PROCEDURES

Instrumentation. Laser-induced lifetimes of Eu(III) in insulin were obtained with an automated transient signal averager system (Alameda et al., 1981; Birge, 1983) (see Figure 3). Short pulses, 1.2 μs in duration, were generated with a Chromatix CMX-4 flashlamp-pumped dye laser using Rhodamine 6G perchlorate as the laser dye. The laser pulses were frequency doubled to 297 nm with an intracavity ammonium

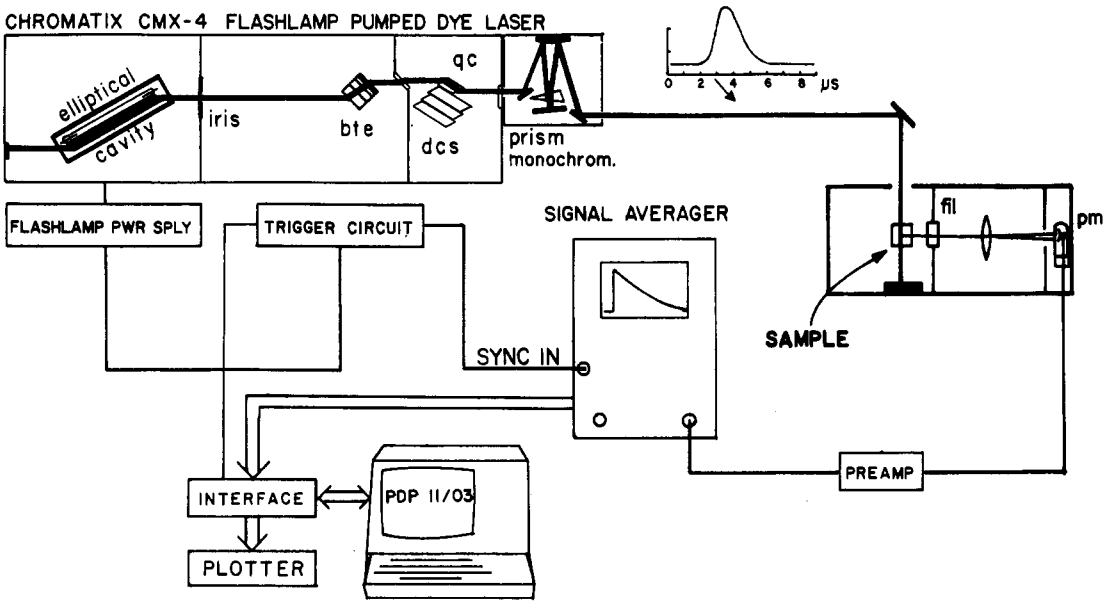

FIGURE 3: Fluorescence lifetime apparatus. Short pulses, 1.2 μs in duration, are generated by a Chromatix CMX-4 flashlamp-pumped dye laser. A birefringent tuning element (bte) selects the wavelength of the laser over the tuning range of the dye. The beam can be passed through a quartz crystal (qc) for the fundamental laser frequency or frequency doubled by an ammonium dihydrogen phosphate crystal (dcs). In the doubling mode, a prism monochromator filters out the fundamental laser frequency. Fluorescence from the sample is passed through a band-pass (560–900-nm) filter (fil) to remove the scattered laser light and refocused onto a photomultiplier tube (PM). The PDP 11/03 computer controls the firing of the laser and triggering of the EG&G P.A.R. Model 4002 signal averager by control pulses sent to the trigger circuit. The data, collected in increments of 5 μs, are transferred to the PDP 11/03 for analysis.

dihydrogen phosphate crystal. A prism monochromator was used to remove the fundamental laser line, leaving only the frequency-doubled component to be passed through the sample. At 90° to the axis of the exciting beam, a second Corning glass band-pass filter (560–900 nm) allowed only the red sample fluorescence to fall on a Hamamatsu R955 red-enhanced photomultiplier tube cooled to −25 °C. Signals were amplified 100-fold by a Princeton Applied Research (P.A.R.) Model 113 amplifier. Collection of transient photocurrent signals were recorded and averaged by a P.A.R. Model 4002 transient signal averager. Typically, 2000–4000 scans (2048 points per scan) were required to produce acceptable signal-to-noise ratios. Data were processed and analyzed with the aid of a Digital Equipment Corp. PDP 11/03 computer interfaced to the transient signal averager (Figure 3).

As a test of the experimental method, fluorescence lifetimes were measured for solutions of Eu(III) in H_2O and D_2O. The data given in Table I and are in good agreement with previously reported lifetimes (Horrocks & Sudnick, 1979).

Quantum efficiency measurements of Zn_2EuIn_6 were performed on a computer-controlled (PDP 11/03) EG&G P.A.R. Model 1205 optical multichannel analyzer (OMA). Details of the electronic interface are described elsewhere (Alameda et al., 1981). An argon ion laser served as the exciting source and was tuned to the UV line at 363.8 nm. With a solution of Rhodamine 6G perchlorate as a fluorescence standard (Q_0 = 0.95; Drexhange, 1976), the dye concentration was adjusted so that the absorbance was almost equal to that of two-zinc(II) insulin bound Eu(III) at the exciting band. A calibrated Schott Glass, Inc., neutral density filter was required between the dye solution and the OMA because the fluorescence intensity was much greater than that of insulin-bound Eu(III).

Determination of the spectral overlap integral in eq 6 for the Eu(III) insulin was performed with the aid of a computerized emission spectrometer developed for this experiment. Sample illumination was from a 100-W Hg–Xe arc lamp. The output was first filtered by a Corning glass (240–400 nm) UV band-pass filter and then sent through a saturated $CuSO_4$ solution to remove any residual red light. Each spectrum was obtained by averaging the measured intensity 45 times at each wavelength. The absorption spectrum of the Co(II) fluorescence acceptor was obtained on a Varian Cary-17 absorption spectrometer.

Table I: Fluorescence Lifetimes of Eu(III) and Insulin-Bound Eu(III) in H_2O and D_2O

	τ_{obsd} (μs)	
	H_2O	D_2O
$Eu(NO_3)_3$	102.8	1993.3
Zn_2EuIn_6	353.0	1163.0
$Zn_2Eu_3In_6$	346.4	1133.5
Eu_3In_6	343.2	1125.6
	τ_b (μs)	% H_2O
Zn_2EuIn_6	420.1	75
Zn_2EuIn_6	543.7	50
Zn_2EuIn_6	741.6	25

Materials. Insulin (bovine, crystalline) was purchased from Sigma Chemical Co. (lot 49C-0197). Chelex 100 (100–200 mesh, Na form) was purchased from Bio-Rad Laboratories. Europium nitrate (99.9 atom % Eu) was purchased from Research Organic/Inorganic Chemical Corp. Deuterium oxide (99.9 atom % D) was purchased from Aldrich Chemical Co. Metal-free sulfuric acid (96%, Supra pure) was purchased from Matheson Coleman and Bell.

Methods. Insulin solutions (5 mg/mL) were prepared by a modification of the method of Dunn et al. (1980). The solutions were prepared in distilled H_2O in the absence of buffer and passed over a Chelex 100 column at pH 10.0, rather than at pH 8.0. Stock solutions of $Eu(NO_3)_3$ and $ZnSO_4$ were prepared (both 1×10^{-2} M) in H_2O and D_2O; a 1×10^{-2} M $CoSO_4$ solution was prepared in D_2O. For measurements in H_2O, metal-free insulin was dissolved in H_2O, the pH was adjusted to 8.0 with dilute metal-free H_2SO_4, and the appropriate metal ions were added. Stoichiometries are based on the weight of lyophilized insulin and the volumes and concentrations of metal ion solutions. The results of sedi-

mentation equilibrium studies (Milthorpe et al., 1977) indicate that at the concentrations used in these studies insulin is present almost exclusively as a hexamer. Because Co(II) is known to replace Zn(II) in insulin (Hudecek et al., 1979), we are confident that insulin is hexameric when there are 2 mol of Zn(II) or Co(II) per 6 mol of insulin monomer. Confirmation of the Eu(III) stoichiometries is provided by the single-exponential decay of Eu(III) fluorescence for stoichiometries of less than three Eu(III) ions to one hexamer. Any free Eu(III) would yield a biexponential decay with a fast-relaxing component, which is observed for stoichiometries of greater than three Eu(III) ions per hexamer.

For measurements in D_2O, metal-free insulin was dissolved in D_2O and the solution pH adjusted to 8.0 with dilute metal-free H_2SO_4. Next, the appropriate metal ions were added, and the sample was lyophilized and redissolved in D_2O to ensure complete removal of H_2O. (Subsequent lyophilizations and dissolution resulted in no measurable increase in the fluorescence lifetime; hence, H_2O removal was judged complete.) Solutions of $Eu(NO_3)_3$ in D_2O were prepared by rotary evaporation of the solution and redissolution in D_2O, repeated 3 times.

Results and Discussion

The fluorescence lifetimes of $Eu(NO_3)_3$ measured in H_2O and D_2O (Table I) are in good agreement with values previously reported (Horrocks & Sudnick, 1979). The emission quantum yield of Zn_2EuIn_6 was observed to be 0.08 ± 0.02 for excitation at 363.8 nm. The relatively large error bar includes error sources associated with the correction procedures outlined below. It should be noted, however, that the quantum yield required for the rigorous application of eq 5 is not Q^{364} but Q^{578}, the quantum yield of emission for excitation into the $^5D_0$ level. The proximity of this excitation wavelength to the prominent emission bands (see Figure 5) precluded accurate measurement of Q^{578}. There is no a priori reason to expect a wavelength-dependent quantum yield, and in fact, a previous study of Tb(III) emission indicated nearly identical quantum yields of emission for excitation throughout the f–f absorption spectrum (Horrocks & Collier, 1981). We predict that a wavelength-independent quantum yield of emission is a characteristic of the lanthanide ions and that our measurement of Q^{364} is a reasonable estimate of the quantum yield for excitation into the acceptor level at 578 nm. Because a large fraction (81.7%) of the incident light is absorbed by the Eu(III)-free two-zinc(II) insulin, the difference in absorbance at 364 nm with and without Eu(III) was determined. This measurement allowed calculation of the fraction of incident light actually absorbed by Eu(III). However, tyrosine present in insulin also absorbs and reemits a portion of the incident light. To correct for the amount of light reabsorbed by the Eu(III), the integral of the tyrosine fluorescence was determined with and without Eu(III). Because Zn(II) is present in the system, addition of Eu(III) is expected to cause little or no change in the hexamer structure. This prediction justifies the use of the above correction as an estimate of the amount of incident light energy actually absorbed by Eu(III).

The emission spectrum of Eu(III) in D_2O is shown in Figure 4. The emission spectra of $Zn_2Eu_3In_6$ and Zn_2EuIn_6 in D_2O are shown in Figure 5. The latter of these spectra (Figure 5b) combined with the absorption spectrum of $CoZnIn_6$ (Figure 6) were employed in the calculation of J, the spectral overlap integral [see Alameda (1981)].

Fluorescence lifetimes of Zn_2EuIn_6 samples prepared in H_2O and D_2O are summarized in Table I. An analysis using eq 3 indicates that the number of coordinated OH groups is 4.0 ± 1.0. Analysis of the data from Table I indicates that the lifetimes follow very closely the bimolecular quenching mechanism (Krupp & Windsor, 1965, 1966):

$$\tau_{D_2O}/\tau_b = 1 + bk_{H_2O}/(k_R + k_X + k_{D_2O}) \quad (7)$$

where τ_b is the observed lifetime of the mixed H_2O–D_2O solution and b the percentage of H_2O in D_2O. Agreement with

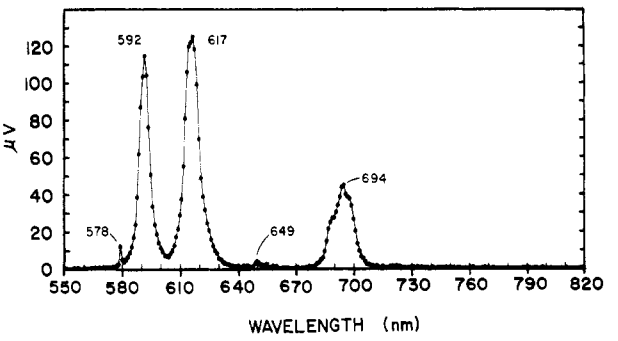

FIGURE 4: Emission spectrum of Eu(III) in D_2O, representing the average of 50 separate scans. The observed fluorescence peaks are assigned as follows: 578 nm, $^5D_0$–$^7F_0$; 592 nm, $^5D_0$–$^7F_1$; 617 nm, $^5D_0$–$^7F_2$; 649 nm, $^5D_0$–$^7F_3$; 694 nm, $^5D_0$–$^7F_4$.

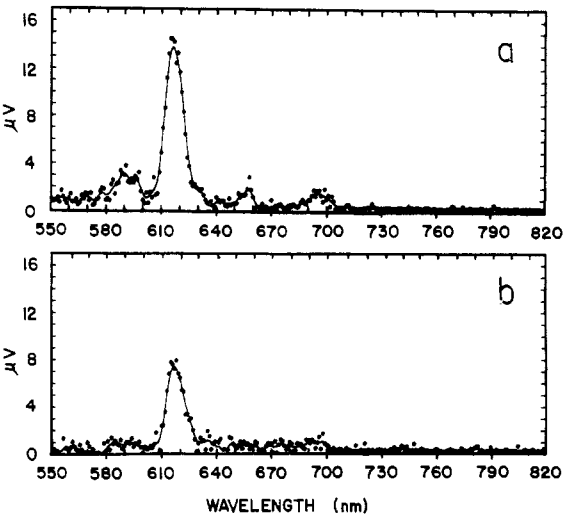

FIGURE 5: Emission spectra of (a) $Zn_2Eu_3In_6$ and (b) Zn_2EuIn_6 in D_2O. The broad tyrosine fluorescence has been subtracted out in both spectra. Sample concentrations were 1 mM in insulin hexamer. Note the $^5D_0$–$^7F_2$ transition is much greater in intensity than that observed for the $^5D_0$–$^7F_1$ band of Eu(III) in D_2O, a characteristic of Eu(III) chelates.

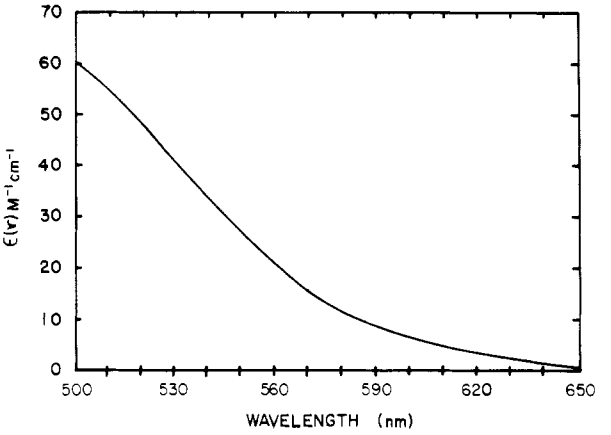

FIGURE 6: Absorption spectrum of $CoZnIn_6$ obtained by using a blank of metal-free insulin in the reference beam. This spectrum is required for the calculation of the spectral overlap integral, J (see text).

the bimolecular quenching mechanism supports the conclusion that quenching of the Eu(III) fluorescence is directly proportional to the number of coordinated OH groups (Kropp & Windsor, 1965, 1966).

To determine the number of similar Ca(II) binding sites in the center of the hexamer, we compared the fluorescence lifetimes of Zn_2EuIn_6, $Zn_2Eu_3In_6$, $Zn_2Eu_4In_6$, and $Zn_2Eu_5In_6$. The fluorescence lifetimes of Zn_2EuIn_6 and $Zn_2Eu_3In_6$ were found to be virtually identical. Least-squares fits of the data are given in Table I. The fit ($r^2 > 0.99$) to a single decaying exponential precludes the possibility of a significant amount of unbound Eu(III). Addition of a fourth or fifth equivalent of Eu(III) results in the presence of a fast-relaxing fluorescence component. Because loosely bound Ca(II) coordination sites are present on the surface of the hexamer (Emdin et al., 1980), the excess Eu(III) is free to come in contact with other energy acceptors such as –OH (Kropp & Windser, 1966) on the surface of the insulin. The observed short relaxation time (approximately 100 μs) is consistent with this type of deexcitation. In order to determine if Eu(III) was bound at Ca(II) binding sites, excess Ca(II) was added to a $Zn_2Eu_3In_6$ sample. The Eu(III) fluorescence developed a fast-relaxing component much the same as in the experiment with excess Eu(III). When excess Zn(II) ions were added to $Zn_2Eu_3In_6$, a negligible change in the Eu(III) fluorescence lifetime was observed, and no fast relaxing component was observed. These results strongly suggest that Eu(III) and Ca(II) compete for the same binding sites.

The Zn(II)–Co(II) distance was determined with $Co_2Eu_3In_6$ and $ZnCoEuIn_6$ samples, where Co(II) replaces Zn(II) (Hudecek et al., 1979) and partially quenches the Eu(III) fluorescence. Introduction of 1 equiv of Co(II) into an insulin sample containing 1 equiv each of Eu(III) and Zn(II) resulted in a measured fluorescence intensity ratio $(I/I_0)_{measd}$ of 0.535. Thus, for a measured fluorescence intensity ratio of 0.54, the quenching ratio I_{Co}/I_0 is 0.46. Because two Zn(II) binding sites are present in the insulin hexamer, there is a distribution of Co(II) and Zn(II), in those sites, and a mixture of Zn_2EuIn_6, Co_2EuIn_6, and $ZnCoEuIn_6$ is present. To calculate the Co(II)–Eu(III) [or Zn(II)–Ca(II)] distance, the fluorescence quenching ratio of one Eu(III) by one Co(II) must be known. Assuming the affinity for the Zn(II) binding sites is equal for Zn(II) and Co(II), the quenching ratio, $I_{Co}/I_0 = [ZnCoEuIn_6]/[Zn_2EuIn_6]$, can be estimated from the following relationship:

$$(0.5I_{Co} + 0.25I_0 + 0.25I_{Co_2})/I_0 = (I/I_0)_{measd}$$

If we let $I_0 = 1.00$ (and note that I_{Co} is twice that of I_{Co_2}), then $I_{Co}/I_0 = [(I/I_0)_{measd} - 0.25]/0.625$. As a check, the quenching ratio of $Co_2Eu_3In_6$ to $Zn_2Eu_6In_6$ was measured and found to be 0.25. In a similar manner, a value of $I/I_0 = 0.50$ is calculated, in good agreement with the one Eu(III) species.

Determination of the metal–metal distance can now be undertaken. Evaluation of eq 6 with the data shown in Figures 5 and 6 yields a value for the normalized spectral overlap integral of $J = 5.85 \times 10^{-17}$ M^{-1} cm^3 (Alameda, 1981). The dipole orientation factor κ^2 is known to assume values between 0 and 4, but a value of $\kappa^2 = 2/3$ (random orientation) is generally found to be acceptable for Eu(III)–Co(II) systems (Horrocks et al., 1975). Lastly, the index of refraction for the surrounding medium is assigned a value of 1.33, the value for water, since the cavity separating Eu(III) from Co(II) is open to solvent. Substitution of these values into eq 4 and 5 results in a calculated metal–metal distance of 9.6 ± 0.5 Å. The error is based upon uncertainties of ±25% in the experimental quantum efficiency value.

From these laser-induced Eu(III) fluorescence studies, it apears there are three symmetrically oriented Eu(III) binding sites near the center of the two-zinc(II) insulin hexamer. The agreement between the Eu(III)–Co(II) distance in these studies and the distance between the B10 histidyl and B13 glutamyl Zn(II) binding sites in Zn_5In_6 (Emdin et al., 1980) strongly suggests that Eu(III) binds to the B13 glutamyl site in $Co_2Eu_3In_6$. Because the Eu(III) fluorescence intensity is decreased upon the addition of Ca(II) but remains constant in the presence of Zn(II), we suggest that these are Ca(II) binding sites in vivo. These sites are formed by the B13 glutamates of adjacent dimers brought together by formation of the hexamer.

The role of Ca(II) in these sites is not clear as the hexamer is stable with only 2 equiv of Zn(II) present. One possibility is that this site is a storage place for Ca(II), providing a nonlabile pool of Ca(II) that is responsible for the regulation of long-term insulin release (Hellman et al., 1976; Klöppel & Bommer, 1979; Andersson & Berggren, 1979). Another possibility is that Ca(II) neutralizes the negative charges present on the B13 glutamate residues of adjacent dimers, thus decreasing the solubility of the hexamer and aiding in the process of crystal formation [cf. Kohnert et al. (1979)]. Future studies on the effect of Ca(II) on insulin crystallization and the metal content of insulin crystals that are isolated from pancreatic β-cells would assist efforts to answer these questions.

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Connective Tissue Activation: Stimulation of Glucose Transport by Connective Tissue Activating Peptide III†

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ABSTRACT: Connective tissue activating peptide III (CTAP III), a human platelet derived growth factor, induced marked stimulation of 2-deoxy[^{14}C]glucose (2dG) uptake in cultures of human synovial cells, chondrocytes, and dermal fibroblasts. Cytochalasin B (2×10^{-5} M) blocked the mediator-induced increase in 2dG uptake; phlorhizin (8×10^{-4} M) partially inhibited this process. When cells were exposed to CTAP III (4×10^{-6} M) for 30 min prior to uptake assay, 2dG uptake was stimulated by 30–110%; greater stimulation (400–800%) occurred following 17–40-h preincubation with the mediator. A 17-h exposure to CTAP III similarly stimulated 3-*O*-methylglucose uptake by over 400%, suggesting that CTAP III stimulated 2dG uptake is mediated via changes in hexose transport. Cycloheximide clearly prevented the 17-h effects of CTAP III on 2dG uptake. Insulin (3×10^{-6} M) stimulated 2dG uptake 40–70% after 30-min preincubation with hormone; little effect was seen after 17-h preincubation. These data suggest that CTAP III stimulates glucose transport shortly after addition to target cells; the major stimulation observed after a 17-h incubation is consistent with the synthesis of new glucose transport protein.

The biologic activities of a human platelet derived growth factor, connective tissue activating peptide III (CTAP III), including promotion of DNA synthesis, stimulation of hyaluronic acid synthetase activity and hyaluronic acid synthesis, stimulation of sulfate incorporation into proteoglycans, and stimulation of prostaglandin E_2 (PGE_2) synthesis, plasminogen activator secretion, increased glucose uptake, and lactate formation (Castor et al., 1977, 1979; Sisson et al., 1980; Castor & Whitney, 1978; Castor & Pek, 1981; Ragsdale et al., 1982). The covalent structure of CTAP III has been reported (Castor et al., 1983b), and the evidence indicates that the N-terminal tetrapeptide and at least one of the two intrachain disulfide bonds are required for the biologic activity of this protein. While the significance of this molecule in human biology is

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