Distance Measurements at the Active Site of Carboxypeptidase A during Catalysis†

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Dedicated to Professor A. E. Braunstein, Moscow, on the occasion of his 70th birthday

ABSTRACT: Both native zinc carboxypeptidase A and the cobalt-substituted derivative rapidly hydrolyze two new series of peptides, Dns-(Gly)$_n$-L-Phe (n = 1, 2, 3, 4) and Dns-(Gly)$_n$-L-Trp (n = 1, 2, 3). In conjunction with cobalt carboxypeptidase these substrates simultaneously afford measurements of kinetic parameters and of distances within and topographical features of the enzyme active center. For zinc carboxypeptidase, values of k_{cat} range from 4×10^1 to 1×10^4 min^{-1} and values of K_m from 8×10^{-5} to 1×10^{-3} M. For the cobalt enzyme, the corresponding k_{cat} values are 1.5–4 times higher and the K_m values are approximately 0.5 lower. Distance measurements are based on electronic energy transfer. The dansyl group serves both as an acceptor of tryptophanyl excitation energy and as its donor to the cobalt atom in a tryptophan–dansyl–cobalt energy-relay system. Energy transfer between enzyme tryptophanyl residues and substrate dansyl groups rapidly identifies the formation and breakdown of enzyme–substrate complexes. Subsequent transfer of energy from the bound dansyl group to the cobalt atom allows calculation of the distance between these moieties when dipole–dipole resonance transfer is the basic process. These distances computed in this manner increase systematically from less than 8 Å for the dipeptides to about 15 Å for the pentapeptides. The values for corresponding members of the phenylalanyl and tryptophanyl series of substrates are in good agreement with each other. They also compare favorably to distances estimated from molecular models assuming an extended peptide to interact with the cobalt atom at the carbonyl oxygen of the bond to be split. The measurement of catalysis related distances in other enzyme active centers should be possible through the incorporation of suitable spectrochemical probes into both the substrate and the enzyme.

Rapid kinetics, designed to resolve mechanistic details of catalysis, and the determination of structures of enzymes are generally performed under widely divergent experimental conditions. However, for the ultimate verification of enzyme mechanisms, the functional characteristics of an enzyme must be related to its structural features through information obtained during the enzymatic reaction (Vallee, 1970).

Toward this end we have employed bovine cobalt carboxypeptidase A, and have synthesized fluorescent N-dansylated peptides which are hydrolyzed rapidly generating environmentally sensitive signals when bound to the enzyme (Latt *et al.*, 1970). These surveyor substrates have been used to report distances between the cobalt atom and the dansyl group within the enzyme active center while simultaneously signalling other aspects of active center topography *as the enzymatic reaction is in progress.*

The fluorescence emission spectrum of the *N*-dansyl blocking group bound to the enzyme overlaps the cobalt absorption spectrum (Figure 1). The resultant energy transfer depends on the sixth power of the distance between the dansyl group and active-site cobalt atom (Förster, 1948, 1965; Stryer and Haugland, 1967) and can be employed to measure their separation. Energy transfer between tryptophanyl residues of the enzyme and the dansyl group of bound substrate, whose spectra satisfy a similar overlap relationship (Figure 1), affords observation of details in the formation and breakdown of the E·S complex. Thus, the cobalt atom, the dansyl group and tryptophanyl residues constitute two donor–acceptor pairs, with the dansyl group playing a dual role: subsequent to excitation of tryptophan, energy is transferred in tandem to the dansyl group and from the dansyl group to the cobalt atom, constituting an energy-relay system, the operation of which is terminated by dissociation of the product after scission of the susceptible bond. In addition to the distance measurements, the probe properties of these species participating in the two segments of the energy relay system can provide syncatalytic[1] contour mapping of the microenvironment of active-center regions of the enzyme and reflect inhibitor interactions at diverse loci (Latt, 1971; Auld and Latt, 1971; D. S. Auld, S. A. Latt, and B. L. Vallee, submitted for publication).

Materials and Methods

Chemicals were reagent or better grade. Preparation of zinc, apo-, and cobalt carboxypeptidase A (Cox), metal-free technique, and absorption spectra measurements were as previously described (Latt and Vallee, 1971). Preparations of cobalt carboxypeptidase on the average contained 1 g-atom of cobalt and 0.006 g-atom of zinc per mole of enzyme as deter-

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[1] This would appear quite important in view of recent evidence (Christen and Riordan, 1970) that aspartate aminotransferase is nitrated only while catalysis is actually proceeding.

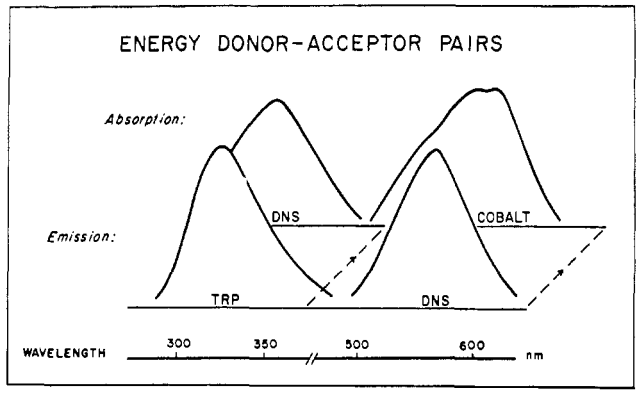

FIGURE 1: Spectral overlap relationships between three moieties of two energy donor–acceptor pairs. Emission spectra: of tryptophan of carboxypeptidase in 1 M NaCl–0.05 M Tris, pH 7.5, 25°, following excitation at 290 nm (left front), and of dansylated dipeptides bound to cadmium carboxypeptidase, obtained in the same buffer, following excitation at 290 nm (right front). Absorption spectra: of dansyl peptide peaking at about 330 nm ($\epsilon \simeq 4.5 \times 10^3$ M^{-1} cm^{-1}) (left rear) and of cobalt carboxypeptidase at neutral pH with maxima at 555 and 572 nm ($\epsilon \simeq 150$ M^{-1} cm^{-1}) (right rear).

TABLE I: R_F Values of N-Dansylated Peptides and Products of Their Hydrolysis by Carboxypeptidase.[a]

Compound	1-Butanol–Glacial Acetic Acid–Water	Ethanol–NH_4OH
Dns-Gly-L-Phe	0.90 (0.78)[b]	0.65 (0.53)
Dns-$(Gly)_2$-L-Phe	0.80 (0.72)	0.44 (0.43)
Dns-$(Gly)_3$-L-Phe	0.67 (0.57)	0.23 (0.18)
Dns-$(Gly)_4$-L-Phe	0.52 (0.40)	0.12 (0.08)
Dns-Gly-L-Trp	0.89 (0.76)	0.55 (0.54)
Dns-$(Gly)_2$-L-Trp	0.77 (0.70)	0.26 (0.41)
Dns-$(Gly)_3$-L-Trp	0.63 (0.56)	0.14 (0.17)
L-Phenylalanine	0.47	0.14
L-Tryptophan	0.44	0.06

[a] Thin-layer chromatography was done on silica gel (Eastman chromogram sheets 6060 with fluorescent indicator). 1-Butanol–glacial acetic acid–H_2O (8:1:) chromatographed for 10 cm (~3 hr) and ethanol–NH_4OH (25:1) for 12 cm (~2 hr). [b] The parentheses in the table indicate the R_F value for the fluorescent dansyl product of the carboxypeptidase catalyzed peptide hydrolysis. These products were identified as Dns-$(Gly)_n$, where n = 1–4, by comparison to authentic samples. Phenylanine and tryptophan were identified by ninhydrin staining. No evidence for formation of glycine was obtained.

mined by atomic absorption analysis (Fuwa and Vallee, 1963; Fuwa *et al.*, 1964). The preparation of cadmium and manganese carboxypeptidase was similar to that of cobalt carboxypeptidase except that cadmium or manganous ion, 10^{-4} M, was substituted for the cobalt in the dialysis to form the metalloenzyme. The resultant cadmium and manganese enzymes contained less than 0.02 and 0.01 g-atom of zinc per mole of enzyme as determined by atomic absorption analysis and 1 g-atom of a given metal, as determined by emission spectrography (Vallee, 1955; Drum and Vallee, 1970).

Carboxypeptidase concentrations were measured by the absorbance at 278 nm using a Zeiss PMQII spectrophotometer. A molar absorptivity at 278 nm of 6.42×10^4 M^{-1} cm^{-1} was used for carboxypeptidase (Simpson *et al.*, 1963).

Substrates.[2] All starting materials for peptide synthesis were obtained from either Cyclo or Sigma Chemical Co. Dns-Gly-L-Trp, Dns-Gly-Gly-L-Phe, and the Dns$(Gly)_n$ series, where n = 1, 2, 3, or 4, were synthesized from the reaction of Dns-Cl with the appropriate glycyl derivative using a procedure similar to that of Gray (1967).

All other dansyl peptides were synthesized by an activated ester procedure similar to that of Anderson *et al.* (1964). One equivalent of Dns-$(Gly)_n$ and *N*-hydroxysuccinimide was dissolved in cold, dry dimethoxyethane (approximately 25 ml/g of Dns-$(Gly)_n$). One equivalent of dicyclohexylcarbodiimide was added to the solution and the reaction mixture stirred at 4° in the dark for at least 24 hr. A solution of 1.2 equiv of either L-phenylalanine or L-tryptophan and at least 2 equiv of $NaHCO_3$ in a 1:1 mixture of dimethoxyethane and water was made in a side-arm flask. The dicyclohexylurea was removed from the *N*-hydroxysuccinimide ester by suction filtration, the filtrate mixing with the above well-stirred solution. The coupling reaction appeared to be instantaneous in all cases, as judged by thin-layer chromatography. The pH of the solution was adjusted to 6, the solution refiltered and dimethoxyethane removed by rotary evaporation. The product was extracted into ethyl acetate. The ethyl acetate extract was washed several times with water, made anhydrous, and evaporated to dryness.

When necessary, the product was recrystallized from water and ethanol. The products so prepared ran as single spots on silica gel using either 8:1:1 1-butanol–glacial acetic acid–water or 25:1 ethanol–NH_4OH and gave the desired product upon hydrolysis (Table I). Ninhydrin analysis on an autoanalyzer showed that less than 0.5% free amine was present in the starting materials, yielding 97–100% of the expected phenylalanine or tryptophan upon enzymatic hydrolysis. Concentrations of the dansyl peptides were calculated from absorbance measurements at 330 nm in 1 M NaCl–0.05 M Tris (pH 7.5), using an ϵ of 4.5×10^3 M^{-1} cm^{-1}, based on dry weight.

Peptidase Activity. Assays were performed in 1 M NaCl, 0.05 M Tris, or 0.02 M Veronal, pH 7.5, 25°. For peptide concentrations greater than 2×10^{-5} M the amount of the unblocked amino acid formed on hydrolysis was determined by either of two automated ninhydrin methods described previously (Auld and Vallee, 1970a,b).

Steady-state fluorescence assay of the substrates Dns-$(Gly)_n$-L-Trp (n = 1, 2, 3) at low ratios of enzyme to substrate was based on the increase in tryptophan fluorescence, subsequent to scission of the bond between glycine and tryptophan (Latt *et al.*, 1972). Ninhydrin and fluorescence assay mixtures containing cobalt carboxypeptidase were 10^{-4} M with respect to cobaltous ion to insure at least 99% formation of the cobalt enzyme as judged by its stability constant (Coleman and Vallee, 1961).

Stopped-flow fluorescence assays were performed at high and comparable concentrations of enzyme and substrate,

[2] The abbreviations used will be: Dns or dansyl, 5-dimethylaminonaphthalene-1-sulfonyl. [(CPD)Zn] or [(CPD)Co] refers to the respective carboxypeptidases A where (CPD) represents apoenzyme and the brackets indicate the firm binding of Zn or Co to it. Hepes refers to *N*-2-hydroxyethylpiperazine-*N*-2′-ethanesulfonic acid.

utilizing a Durrum-Gibson instrument, equipped with Kel-F connections. The temperature was controlled to ±0.2°. Emission was measured using a band-pass filter peaking at 360 nm (for tryptophan) or a cut off filter excluding light below 430 nm (for the dansyl moiety). Initially, in the first set of distance measurements, the R375 photomultiplier tube supplied by the manufacturer was used. For the second set of measurements, sensitivity was increased by replacing it with an EMI 9526 B photomultiplier tube mounted near the cuvet for end-on observation.

Determination of Stopped-Flow Parameters. Oscilloscope traces of the time course of fluorescence were photographed on Polaroid Type 107 black and white film. The photographs were then traced on Keuffel and Esser Co. Albanese Guide Line 10-5663 millimeter paper. Values of initial curve height and time of its decrease by 50% were obtained from these traces.

Stopped-flow fluorescence measurement of energy transfer also utilized the Durrum-Gibson instrument. Prior to experiments with cobalt carboxypeptidase, the syringes were rinsed with 6 N HCl. The cell block was filled with 1,10-phenanthroline (10^{-3} M) and thoroughly rinsed with dilute HCl, followed by several rinses with metal-free buffer. Solutions of cobalt carboxypeptidase were 1×10^{-4} M with respect to free Co(II) ions prior to mixing with substrate.

Energy transfer was measured subsequent to excitation at 285 nm. Fluorescence changes due to E·S complex formation were observed against a nearly constant background of low intensity due to enzyme and free substrate or product. The magnitudes of background and buffer blanks could be measured independently, and changes due to substrate tryptophanyl fluorescence were readily corrected for in that series of substrates. These corrections, amounting to less than 10% of the dansyl signal and an average of 50% of the tryptophan signal, were applied based on fluorescence signals following hydrolysis and the known relative contributions of species present. The dansyl products of hydrolysis are bound orders much more weakly than is the substrate. Therefore dissociation of the dansyl product after scission of the susceptible bond terminates the energy-relay system.

Relative fluorescence efficiencies, F', were computed to render this estimate insensitive to minor differences in substrate K_m or energy transfer efficiency from enzyme tryptophans to substrate dansyl for the zinc and cobalt enzyme (eq 1). In each case, the measured fluorescence intensity, F, of the dansyl group bound to each metalloenzyme was normalized by the fractional quenching of enzyme tryptophan fluorescence in the E·S complex, ϕ

$$F = \phi F' \tag{1}$$

$$\phi = T' \times \frac{[ES]}{[E_T]} \tag{2}$$

where $[ES]/[E_T]$ is the fraction of enzyme complexed with substrate and T' is the efficiency of quenching of enzyme tryptophan fluorescence by bound substrate.

Energy transfer, T, from the dansyl group of the substrate in the E·S complex to the cobalt atom of the enzyme was calculated from the relative fluorescence efficiency F'_{Co}/F'_{Zn} of the dansyl moiety of substrate when bound to either one of the two enzymes.

$$1 - T = F'_{Co}/F'_{Zn} \tag{3}$$

The ratio of donor–acceptor separation, R, to the critical distance for 50% efficient energy transfer, R_0, was calculated from the equation (Förster, 1948, 1965)

$$1 - T = 1/[1 + (R_0/R)^6] \tag{4}$$

R_0 was obtained from the relationship

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

where Q is the donor quantum yield in the absence of energy transfer, n is the index of refraction of the medium separating the donor and acceptor, κ^2 is a dipole–dipole orientation factor, and J is the spectral overlap integral

$$J = \frac{\int \epsilon_\lambda F_\lambda \lambda^4 d\lambda}{\int F_\lambda d\lambda} \text{ cm}^3 \text{ M}^{-1} \tag{6}$$

where ϵ_λ is the molar extinction coefficient of the acceptor, in units of $\text{cm}^{-1}\text{ M}^{-1}$, and F_λ is the donor fluorescence.

Fluorescence quantum yields, Q, were determined relative to values of 0.06 for Dns-Gly (Chen, 1967b) and 0.13 for L-Trp (Chen, 1967c). Determination of dansyl quantum yields for substrates bound to zinc carboxypeptidase was based on measurements of dansyl fluorescence obtained from excitation either at 285 nm or directly, at 330 nm, both adjusted for the fractional saturation of enzyme by substrates. The amount of E·S complex formed was calculated from the steady-state K_m value. Measurements based on excitation at 285 nm were subject to less experimental error, due to their high signal to noise ratio, but could be used only to derive quantum yields relative to the dipeptides. A value of 0.08 for the dansyl quantum yield of bound dipeptides obtained by excitation at 330 nm was used as the standard for comparison with the longer peptides.

Fluorescence spectra were measured using the ratio mode, on a Hitachi MPF 2-A spectrofluorometer, kindly made available by Dr. Elkan R. Blout. Emission spectra were corrected for monochromator efficiency and response of the R-106 phototube according to the method described by Chen (1967a). Fluorescence spectra of the cadmium carboxypeptidase Dns-Gly-L-Trp or Dns-Gly-L-Phe complexes were obtained by exciting at 290 nm to excite bound substrate selectively.

Results

The series of dansylated substrates for carboxypeptidase A have the kinetic and spectral characteristics required to probe active-center topography during rapid catalysis. Carboxypeptidase cleaves Dns-$(Gly)_n$-L-Trp (n = 1, 2, 3) and Dns-$(Gly)_n$-L-Phe (n = 1, 2, 3, 4) at the C-terminal peptide bond at rates comparable to those of benzyloxycarbonyl and benzoyl substrates previously investigated (Snoke and Neurath, 1949; Auld and Vallee, 1970a). For all substrates except the tripeptides, reciprocal plots are linear over 10- to 1000-fold substrate concentration ranges. Such plots of tripeptides are curved above 10^{-3} M. The stopped-flow studies to be described (*vide infra*) were performed at concentrations of these substrates well below 10^{-3} M. Maximal activities of zinc carboxypeptidase toward the two series of dansyl peptides vary from 4×10^1 min^{-1} to greater than 1×10^4 min^{-1} (Table II).

TABLE II: Carboxypeptidase-Catalyzed Hydrolysis of Dansyl Peptides: Michaelis–Menten Parameters.[a]

Substrate	$K_m \times 10^4$ M		k_{cat} (min^{-1})	
	[(CPD)Zn]	[(CPD)Co]	[(CPD)Zn]	[(CPD)Co]
Dns-Gly-L-Phe	0.8	0.4	43	170
Dns-Gly-L-Trp	1.1	0.5	62	252
Dns-Gly-Gly-L-Phe	6.0	4.2	11,000	22,500
Dns-Gly-Gly-L-Trp	13.0	7.0	11,600	16,000
Dns-Gly-Gly-Gly-L-Phe	5.7	3.3	4,200	8,200
Dns-Gly-Gly-Gly-L-Trp	13.0	5.6	3,900	5,900
Dns-Gly-Gly-Gly-Gly-L-Phe	5.7	3.2	1,100	2,600

[a] Assays employed 1 M NaCl–0.05 Tris or Hepes, pH 7.5, 25°. Concentration of dipeptides ranged from 0.3×10^{-4} to 3×10^{-4} M and that of tri-, tetra-, and pentapeptides from 0.1×10^{-3} to 1×10^{-3} M.

The maximal activities of cobalt carboxypeptidase catalyzed hydrolysis of the dansyl peptides are 1.5- to 4-fold greater than those of the zinc enzyme, while K_m's are somewhat lower for the cobalt than for the zinc enzyme (Table II).

Spectral overlap between tryptophan emission and dansyl absorption (Figure 1) affords highly efficient energy transfer between enzyme and substrate at all possible donor–acceptor distances in the E·S complex. Thus, using stopped-flow fluorescence at comparable and high enzyme and substrate concentrations, $\sim 10^{-4}$ M, both the formation and rapid hydrolysis of enzyme–dansyl substrate complexes can be observed. On binding of the dansyl dipeptides to zinc carboxypeptidase, enzyme tryptophan fluorescence is quenched by nearly 30% of the total signal, and substrate dansyl fluorescence is enhanced concomitantly by about 200% of background (Figure 2). Signal amplitude is proportional to the amount of E·S complex formed and the efficiency of energy transfer. The half-time for signal decay, reflecting reduction of substrate concentration by hydrolysis, depends on the ratio of K_m/k_{cat}. The hydrolysis of dansylated tri-, tetra-, and pentapeptides has been examined similarly, and their kinetic behavior is consistent with steady-state data. For the tryptophan fluorescence of this zinc enzyme–dipeptide substrate complex the apparent quenching efficiency, T' (eq 2), is 60%. For complexes with the successively longer peptides it is estimated to be 100, 40, and 30%, respectively.

For all free peptides used in these experiments, the quantum yield of the dansyl group was 0.06. Upon binding to zinc carboxypeptidase, this value increases only slightly for the di-, tetra-, and pentapeptides, but increases markedly for tripeptides, as evidenced by stopped-flow experiments where the dansyl group is excited directly at 330 nm (Figure 3). Relative to a value of 0.08 for the dipeptides, estimates of bound substrate quantum yield, Q, are also provided by measurement of dansyl emission subsequent to excitation at 285 nm and energy transfer from the enzyme. These determinations of Q utilize values of either steady-state K_m or

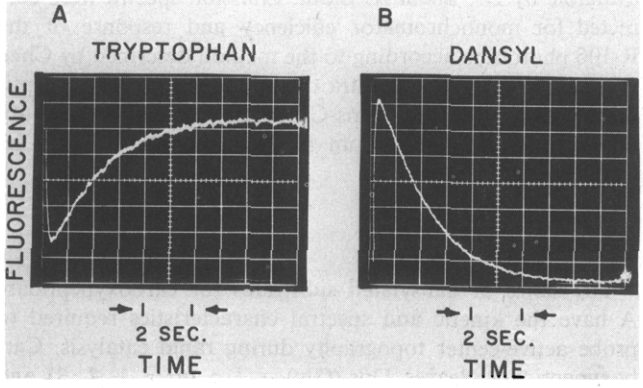

FIGURE 2: Enzyme tryptophan (A) and substrate dansyl (B) fluorescence during the time course of zinc carboxypeptidase catalyzed hydrolysis of Dns-Gly-L-Phe. Equal volume solutions of substrate and of enzyme, both 2.5×10^{-4} M, in 1 M NaCl–0.02 M Tris, pH 7.5, 25°, were mixed and the fluorescence of either tryptophan (A) or dansyl (B) was measured as a function of time under stopped-flow conditions in parallel samples, as shown by the oscilloscope tracings. Excitation was at 285 nm. Scale sensitivities for (A) and (B) are 100 mV/div. The existence of the E·S complex is signalled either by (A) the *suppression* of enzyme tryptophan fluorescence (quenching by the dansyl group) or (B) *enhancement* of the substrate dansyl group fluorescence (energy transfer from enzyme tryptophan).

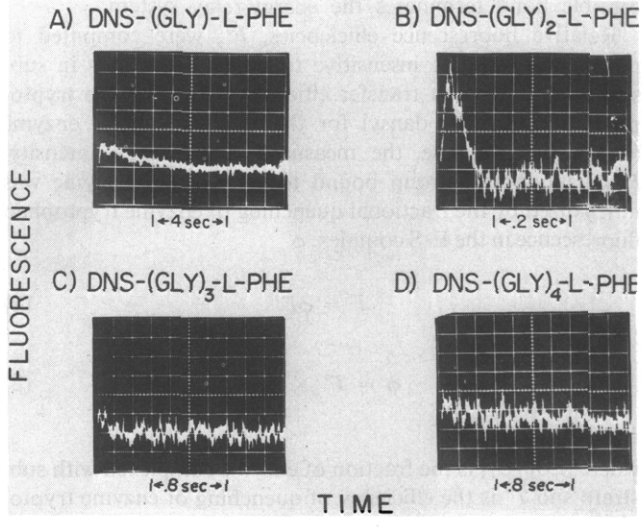

FIGURE 3: Effect of dansyl fluorescence efficiency of substrate binding to zinc carboxypeptidase. Dns-(Gly)$_n$-L-Phe and zinc carboxypeptidase solutions were mixed as described in Figure 2. Substrate concentrations were 1.25×10^{-4} M for the dipeptide and 3.75×10^{-4} M for the other peptides. The enzyme concentration was 1.25×10^{-4} M in all the experiments. Excitation was at 330 nm. The emission measured was that of the dansyl. The scale sensitivity for all tracings was 5 mV/division. Base-line signals were (A) 23, (B) 29, (C) 30, and (D) 31 mV.

TABLE III: Quantum Yield of Substrate Dansyl Bound to Zinc Carboxypeptidase.[a]

Substrate	I[b]	II[c,d]	III[c,e]	R_0 (A°)
Dns-Gly-L-Phe	0.08	0.08	0.08	16.5
Dns-Gly-L-Trp	0.08	0.08	0.08	16.5
Dns-Gly-Gly-L-Phe	0.52	0.30	0.55	20.5–22.6
Dns-Gly-Gly-L-Trp	0.72	0.32	0.58	20.8–23.8
Dns-Gly-Gly-Gly-L-Phe	0.10	0.14	0.09	16.7–18.0
Dns-Gly-Gly-Gly-L-Trp	0.13	0.25	0.15	17.8–19.9
Dns-Gly-Gly-Gly-Gly-L-Phe	0.11	0.12	0.09	16.7–17.7

[a] 1 M NaCl–0.02 M Tris, pH 7.5, 25°. [b] Determined from excitation at 330 nm, normalized for enzyme saturation calculated from substrate K_m. [c] Relative to a value for Q of 0.08 for the bound dipeptides. [d] Determined from excitation at 285 nm, normalized for enzyme tryptophan quenching. [e] Determined from excitation at 285 nm, normalized for enzyme saturation calculated from substrate K_m.

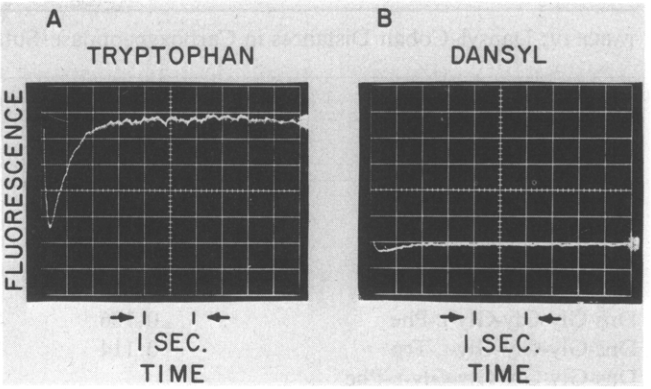

FIGURE 4: Enzyme tryptophan (A) and substrate dansyl (B) fluorescence during the time course of cobalt carboxypeptidase catalyzed hydrolysis of Dns-Gly-L-Phe. Procedure and conditions as in Figure 2. Suppression of tryptophan fluorescence (A) is evidence for the existence of the E·S complex. Quenching of substrate dansyl fluorescence by enzyme cobalt (B) signals energy transfer, and, hence, distance.

enzyme quenching, ϕ, to estimate energy received by the bound dansyl group. The results are summarized in Table III.

Substrate quenching of enzyme fluorescence also identifies formation of the cobalt carboxypeptidase–substrate complexes (Figure 4A). The amplitudes of these signals are similar to those with the zinc enzyme,[3] reflecting comparable binding strengths. The decay of the cobalt carboxypeptidase complexes is more rapid and results from the greater intrinsic activity of that enzyme, observed previously by steady-state assay (Table II).

The broad visible absorption spectrum of cobalt carboxypeptidase (ϵ_{max} ~150 M^{-1} cm^{-1}) (Latt and Vallee, 1971), overlaps the dansyl emission (Figure 1), resulting in a second energy-transfer process, from the dansyl group of bound substrate to the cobalt atom at the active site. Quenching of the dansyl emission is an exquisitely sensitive function of the dansyl–cobalt distance and is complete for the bound dipeptide Dns-Gly-L-Phe (Figure 4B), indicating the close proximity of the dansyl group and of the cobalt atom on formation of this complex. Insertion of additional amino acid residues to elongate the substrate diminishes quenching, consistent with consequent placement of the dansyl group at a greater distance from the cobalt atom. These signals have been normalized (eq 1) for quenching of enzyme tryptophan fluorescence by the dansyl group in the series Dns-$(Gly)_n$-L-Phe to permit computation of relative dansyl fluorescence efficiency, $1 - T$, in the cobalt E·S complex. For the di-, tri-, tetra-, and pentapeptide zinc–enzyme complexes, $1 - T$ increases from an undetectable amount to about 1, 10, and 25%, respectively (Figure 5).[4]

Donor–acceptor separations can be calculated from energy-transfer efficiency, T (eq 3), which determines R/R_0 (eq 4), and the spectral parameters κ^2, n, Q, and J, which determine R_0 (eq 5). For computation of R_0, n is taken to be the index of refraction of the solvent, 1.33. For the dipole–dipole orientation factor, κ^2, a value of 2/3 is used (*vide infra*). The value of the spectral overlap integral, $J = 1.3 \times 10^{-15}$ cm^3 M^{-1}, was calculated from the absorption spectrum of cobalt carboxypeptidase and the dansyl emission spectrum of dipeptides when bound to cadmium carboxypeptidase since the latter is inactive as a peptidase (Coleman and Vallee, 1960) (eq 6). The above values for n, κ^2, and J, together with the various estimates of Q, leads to a range of values for R_0 (Table III).

The experimental values of F'_{Co}/F'_{Zn} used to determine R/R_0 (eq 3) vary from less than 2.2 for the dipeptides to 1.2 for the pentapeptide. Based on these and the calculated values of R_0 (Table III), the cobalt dansyl distance R can be estimated, ranging from less than 8 Å for the dipeptides to nearly 15 Å for the pentapeptide (Table IV). These values increase

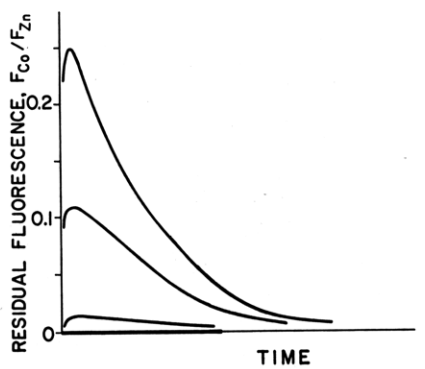

FIGURE 5: Distance-related quenching of peptide dansyl by cobalt in the E·S complex. Stopped-flow fluorescence measurements were performed as described in the caption to Figure 2. See Figure 3 for the composition of enzyme and substrates. Curves of increasing maximal height refer to Dns-Gly-L-Phe, Dns-$(Gly)_2$-L-Phe, Dns-$(Gly)_3$-L-Phe, and Dns-$(Gly)_4$-L-Phe, respectively. The ordinate of the curve represents dansyl fluorescence in the complex of each cobalt carboxypeptidase–substrate complex divided by dansyl fluorescence of the respective zinc carboxypeptidase–substrate complex, normalized for quenching of enzyme fluorescence (eq 1). For presentation on a common, dimensionless abscissa the time axis of each curve has been divided by the half-time of the decay curve.

[3] For a given dansyl substrate, fractional quenching of enzyme fluorescence in the E·S complex, ϕ, averaged about 25% less for cobalt carboxypeptidase compared to the zinc enzyme.

[4] Quenching of dansyl fluorescence is not observed when the paramagnetic but essentially nonchromophoric manganese atom replaces zinc at the active site of the enzyme. However, for the manganese enzyme, the kinetics of hydrolysis for the peptides Dns-$(Gly)_n$-L-Phe (n = 1, 2, 3) are altered. The rate of hydrolysis of the dipeptide is increased while those of the tri- and tetrapeptide are reduced.

TABLE IV: Dansyl–Cobalt Distances in Carboxypeptidase–Substrate Complexes[a,b]

Substrate	Experiment I		Experiment II		R, A° C–P–K Models[c]
	F_{Co}/F_{Zn}	R (A°)	F_{Co}/F_{Zn}	R (A°)	
Dns-Gly-L-Phe	<0.01	<8[d]	<0.01	<8[d]	7
Dns-Gly-L-Trp	<0.01	<8[d]	<0.01	<8[d]	
Dns-Gly-Gly-L-Phe	0.012	9.9–11.0	0.018	10.1–11.3	10
Dns-Gly-Gly-L-Trp	0.028	11.5–13.1	0.019	10.8–12.3	
Dns-Gly-Gly-Gly-L-Phe	0.106	11.7–12.7	0.107	11.7–12.7	13
Dns-Gly-Gly-Gly-L-Trp	0.114	12.5–14.1	0.127	12.9–14.4	
Dns-Gly-Gly-Gly-Gly-L-Phe			0.247	14.1–14.7	16

[a] 1 M NaCl–0.02 M Tris, pH 7.5, 25°. [b] The range of experimental values for the distance, R, reflects that of possible bound substrate quantum yields. [c] Measured on Corey–Pauling–Koltun molecular models from the center of the dansyl group to the cobalt atom assuming the peptide to be in an extended conformation and the metal to bind the oxygen of the C-terminal peptide bond. [d] Based on experimental noise and limits of zinc contamination of the cobalt enzyme.

systematically with peptide-chain length; but values of R for phenylalanyl and tryptophanyl peptides of the same length agree closely. Analogous distances were measured on Corey–Pauling–Koltun molecular models of the peptides when these are in the extended form, employing the center of the dansyl group and the carbonyl atom of the scissile C-terminal peptide bond as fixed points. These values are shown for comparison (Table IV).

Discussion

It is necessary for the present approach that the identity of an essential component of the active center be known, that it be the locus of interaction with a specific region of the substrate and that it exhibit distinctive probe properties which also interact with those of the substrate. Strategic placement of a cobalt atom, a specific energy acceptor, at the active site and the design and synthesis of N-dansylated surveyor substrates satisfy these criteria. These substrates can serve both to donate energy to cobalt, which interacts with the susceptible peptide bond (Vallee *et al.*, 1963; Lipscomb *et al.*, 1970), and to accept energy from tryptophanyl residues of the enzyme. Fluorescence events in the E·S complex are inherently rapid enough that environmental interactions can be reported during the catalytic event.

Fluorescence measurement of transfer of electronic energy has served to detect distances, vicinal features, and changes in local environment (Udenfriend, 1969; Chen *et al.*, 1969). Intermolecular electronic energy transfer due to dipole–dipole interaction depends on fluorophore environment and donor–acceptor distances as discussed by Förster (1948, 1965). Experiments on simple model systems where the dimensions and many parameters are known (Drexhage *et al.*, 1963; Latt *et al.*, 1965; Stryer and Haugland, 1967; Haugland *et al.*, 1969) support this theory, providing a basis for the use of energy transfer in estimating proximity in more complex situations (Conrad and Brand, 1968; Eisinger *et al.*, 1969; Gohlke *et al.*, 1969; Badley and Teale, 1971).

Under the conditions here employed energy transfer from the dansyl group to the cobalt atom signals the distance between the two. Elongation of peptide substrates by progressive introduction of additional amino acid residues allows systematic study of dansyl cobalt distances and other topographic features of the active center while catalysis is proceeding (Figures 3 and 5). Energy transfer between enzyme tryptophanyl residues and the substrate dansyl group reflects regions of the enzyme which interact with this blocking group while demonstrating E·S complex formation, irrespective of the chemical identity of the active-site metal atom (Figures 2A and 4A).

In the present system, energy transfer is measured readily. The low absorptivity of the cobalt atom[5] accounting in part for the low R_0 sets the scale for effective use of energy transfer to estimate R. For the dipeptides, F_{Co}/F_{Zn} is no more than the experimental uncertainty in its estimation, corresponding to an R_0/R of about 2.2. For the longer peptides R_0/R decreases to 1.2 for the pentapeptide, a value at which energy-transfer efficiency becomes particularly sensitive to donor–acceptor separation. The values of R calculated for the present system, while small, are still greater than the 5–7-Å range at which other interactions, such as exchange effects, predominate (Eisinger *et al.*, 1969).

Determination of distances from energy-transfer measurements, which depend on R/R_0, require separate evaluation of R_0. R_0 is proportional to $(\kappa^2 JQ/n^4)^{1/6}$ (eq 5). The exact proportionality depends on theory (Förster, 1948, 1965) from which absolute transfer rates are calculated. While there may be uncertainty in this estimation, it is unlikely that the proportionality varies from one to the next member of this series of compounds.

The index of refraction, n, for the medium separating the donor and acceptor must be estimated. It is improbable that the refractive index would deviate sufficiently from that of the solvent, 1.33, to appreciably affect distance estimates.

The donor–acceptor overlap integral, J, is calculated from the absorption spectrum of the acceptor and the emission spectrum of the donor. A value of $J = 1.3 \times 10^{-15}$ cm^3 M^{-1} is obtained using the absorption spectrum of the free cobalt enzyme and the emission spectrum of the cadmium carboxypeptidase–substrate complexes. Estimation of J is greatly aided by the band width of the absorption spectrum of cobalt carboxypeptidase which exceeds that of the dansyl emission spectrum (Figure 1). Shifts in the spectra of the cobalt en-

[5] At the enzyme concentrations used, the inner filter effect due to reabsorption of dansyl fluorescence by cobalt is insignificant.

zyme measureable for complexes with slowly hydrolyzed nondansylated substrates would alter this value of J by less than 25%, corresponding to a change in R of only 4%. Similarly, the influence of shifts in the bound peptide dansyl emission on J would be expected to be very small, since the range of wavelengths of dansyl emission in different environments (Chen, 1967b) would still guarantee extensive overlap with the cobalt absorption spectrum.

The donor quantum yield, Q, is determined relative to known standards exhibiting similar spectra. For the bound, dansylated peptides, Q was determined relative to the free peptide value of 0.06. Difficulties in measurement of Q during the rapid hydrolysis of the longer peptides together with variations inherent in the different methods used to estimate Q could be responsible for an error as high as 50% in this quantity, but this would be equivalent maximally to an uncertainty in the average value of R_0 of less than 10%.

Assignment of the probable value of the dipole–dipole orientation factor, κ^2, has consistently posed problems in the determination of R_0 (see references cited). Inherently it is the parameter most likely to result in error. κ^2 can range from 0, when all vectors are mutually orthogonal, to 4, when all vectors are parallel. The random average for donor–acceptor orientation is 2/3. Clearly, when κ^2 assumes extreme values, *i.e.*, either 0 or 4, very large errors could ensue. In the present study, cobalt(II) was chosen as an energy acceptor, in large measure, to obviate these extremes. It has the great advantage that the nearly triply degenerate visible absorption transitions of cobalt(II) (Ballhausen, 1962) limit the possible range of κ^2 values from about 1/3 to 4/3, with 2/3 still remaining the random average.[6]

The distances between the cobalt atom, acting at the susceptible peptide bond, and the dansyl group of bound substrates, as determined experimentally by means of energy transfer, are within the limits of those measured on CPK models of such peptides, assumed to be in an extended conformation (Table IV). Further, the increase in distance as a function of chain lengths of the peptides is internally consistent for both sets of substrates, and the results obtained for corresponding members of the two sets of peptides agree well. Yet, for the reasons outlined, the relative rather than the absolute correspondence of data obtained by these two types of measurements are being emphasized.

The dansyl–cobalt energy-transfer measurements determine the radii of arcs about the cobalt atom along which the dansyl group might lie. The interaction of these arcs with the enzyme surface would define the regions surveyed by the dansyl peptide substrates. Substrate orientation within such contour regions remains to be determined.[7]

Energy transfer between the tryptophanyl residues of the enzyme and the dansyl group of the substrate is highly efficient. The critical distance for 50% quenching, R_0, is about 20 Å. This distance is of the same magnitude or greater than that expected for tryptophanyl residues from the active-site region (Lipscomb *et al.*, 1970). However, as peptide chain length increases there is a decrease in the degree of tryptophan quenching. The distances between specific tryptophanyl residues of the enzyme and the dansyl blocking groups of the substrate might be determined if the fluorescence of each tryptophanyl residues could be observed separately.

The quantum yield of the dansyl group of the bound substrate can serve as an additional index of the active-center topography. Thus, a marked increase in quantum yield is observed upon binding of dansylated tripeptides to zinc carboxypeptidase (Figure 5 and Table III). In contrast, the increase in quantum yield for bound dansylated di-, tetra-, and pentapeptides is only slight. Increases of quantum yield of dansylated compounds in organic solvents, accompanied by blue shifts of the fluorescence, are well known (Chen, 1967b). Similar fluorescence changes have been considered to reflect interactions of various chromophores with a nonpolar environment (*e.g.*, Weber and Laurence, 1954; Stryer, 1965; Edelman and McClure, 1968; Turner and Brand, 1968).

The present approach yields information on the active site of carboxypeptidase A which can be integrated with that obtained either by purely kinetic or by solely structural measurements. Owing to the sensitivity of the fluorescence method, the distance determinations can be performed at very low concentrations of enzyme and substrate while rapid catalysis is proceeding. Moreover, the distance scale is set by the spectral properties of donor and acceptor and can be detailed and extended by means of other resonance measurements. Selective placement of donor and acceptor groups at various strategic positions on the enzyme surface may even allow the measurement of conformational movements. The wide range of peptide and ester substrates of carboxypeptidase now available (Abramowitz *et al.*, 1967; Auld and Holmquist, 1972) provides considerable opportunity to map the interaction of homologous series of substrates. The assessment of catalysis related distances in other enzyme active centers should be possible through the incorporation of suitable spectrochemical probes into both the substrate and the enzyme.

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References

Abramowitz, N., Schechter, I., and Berger, A. (1967), *Biochem. Biophys. Res. Commun. 29*, 862.

Anderson, G. W., Zimmerman, T. E., and Callahan, F. M. (1964), *J. Amer. Chem. Soc. 86*, 1839.

Auld, D. S., and Holmquist, B. (1972), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 31*, 435.

Auld, D. S., and Latt, S. A. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 1078.

Auld, D. S., and Vallee, B. L. (1970a), *Biochemistry 9*, 602.

Auld, D. S., and Vallee, B. L. (1970b), *Biochemistry 9*, 4352.

Badley, R. A., and Teale, F. W. J. (1971), *J. Mol. Biol. 58*, 567.

Ballhausen, C. J. (1962), Introduction to Ligand Field Theory, New York, N. Y., McGraw-Hill.

[6] For the case of two dipoles, $\bar{\mu}_1$ and $\bar{\mu}_2$, normalized to unit length, $\kappa^2 = (\bar{\mu}_1 \cdot \bar{\mu}_2 - 3(\bar{\mu}_1 \cdot \bar{L})(\bar{\mu}_2 \cdot \bar{L}))^3$ where $\bar{L}$ is a unit vector with the direction of the line between the two dipoles. Uncertainty in $\bar{\kappa}^2$ arises because of difficulty in determining $(\bar{\mu}_1 \cdot \bar{L})(\bar{\mu}_2 \cdot \bar{L})$. With an energy acceptor composed of three orthogonal dipoles, *i.e.*, possessing a triply degenerate transition, κ^2 can be evaluated by summing the three contributions from the components of a vector $[\bar{\mu}_1 = 1/(3)^{1/2}(\bar{i} + \bar{j} + \bar{k})]$. Then, $\kappa^2 = 1/3[1 + 3(\bar{\mu}_2 \cdot \bar{L})^2]$. As $(\bar{\mu}_2 \cdot \bar{L})^2$ ranges from 0 to 1 with a mean value of 1/3, $\bar{\kappa}^2$ ranges from 1/3 to 4/3 with a mean value of 2/3. Thus, the maximum uncertainty in choosing a value for $\bar{\kappa}^2$ of 2/3 is reduced to a factor of 2, and its sixth root is 1.12. Since it is improbable that the orientations are all absolutely fixed at extremes, the likely resultant error in R_0 is much less. For the reasons described the visible spectrum of the cobalt atom in cobalt carboxypeptidase approximates such an energy acceptor with multiple, orthogonal directions of polarization.

[7] There are small but consistent differences in the efficiency with which bound substrate dansyl quenches enzyme tryptophanyl fluorescence of the zinc and cobalt enzymes.

Chen, R. F. (1967a), *Anal. Biochem. 20*, 339.
Chen, R. F. (1967b), *Arch. Biochem. Biophys. 120*, 609.
Chen, R. F. (1967c), *Anal. Lett. 1*, 35.
Chen, R. F., Edelhoch, J., and Steiner, R. F. (1969) *in* Physical Principles and Techniques of Protein Chemistry, Leach, S. J., Ed., New York, N. Y., Academic Press, Chapter 4.
Christen, P., and Riordan, J. F. (1970), *Biochemistry 9*, 3025.
Coleman, J. E., and Vallee, B. L. (1960), *J. Biol. Chem. 235*, 390.
Coleman, J. E., and Vallee, B. L. (1961), *J. Biol. Chem. 236*, 2244.
Conrad, R., and Brand, L. (1968), *Biochemistry 7*, 777.
Drexhage, K. H., Zwick, M. M., and Kuhn, H. (1963), *Ber. Bunsenges. Phys. Chem. 67*, 62.
Drum, D. E., and Vallee, B. L. (1970), *Biochemistry 9*, 4078.
Edelman, G. M., and McClure, W. O. (1968), *Accounts Chem. Res. 1*, 65.
Eisinger, J., Feuer, B., and Lamola, A. A. (1969), *Biochemistry8*, 3908.
Förster, T. (1948), *Ann. Phys. 2*, 55.
Förster, T., (1965), *in* Modern Quantum Chemistry, Part III, Sinanoglu, O., Ed., New York, N. Y., Academic Press, p 93.
Fuwa, K., Pulido, P., McKay, R., and Vallee, B. L. (1964), *Anal. Chem. 36*, 2407.
Fuwa, K., and Vallee, B. L. (1963), *Anal. Chem. 35*, 942.
Gohlke, J. R., Conrad, R. H., and Brand, L. (1969), Abstracts, 158th National Meeting of the American Chemical Society, New York, N. Y., Sept, p 61.
Gray, W. R. (1967), *Methods Enzymol. 11*, 137.
Haugland, R. P., Yguerabide, J., and Stryer, L. (1969), *Proc. Nat. Acad. Sci. U. S. 63*, 23.
Latt, S. A. (1971), Ph.D. Thesis, Harvard University.
Latt, S. A., Auld, D. S., and Vallee, B. L. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 1383.
Latt, S. A., Auld, D. S., and Vallee, B. L. (1972), *Anal. Biochem.* (in press).
Latt, S. A., Cheung, A. H. T., and Blout, E. R. (1965), *J. Amer. Chem. Soc. 87*, 995.
Latt, S. A., and Vallee, B. L. (1971), *Biochemistry 10*, 4263.
Lipscomb, W. N., Reeke, G. N., Jr., Hartsuck, J. A., Quiocho, F. A., and Bethge, P. H. (1970), *Phil. Trans. Roy. Soc. London, Ser. B 257*, 177.
Simpson, R. T., Riordan, J. F., and Vallee, B. L. (1963), *Biochemistry 2*, 616.
Snoke, J. E., and Neurath, H. (1949), *J. Biol. Chem. 181*, 789.
Stryer, L. (1965), *J. Mol. Biol. 13*, 482.
Stryer, L., and Haugland, R. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 719.
Turner, D. C., and Brand, L. (1968), *Biochemistry 7*, 3381.
Udenfriend, S. (1969), Fluorescence Assay in Biology and Medicine, Vol. 2, New York, N. Y., Academic Press.
Vallee, B. L. (1955), *Advan. Protein Chem. 10*, 317.
Vallee, B. L. (1970), *VIII Intern. Congr. Biochem.*, 158.
Vallee, B. L., Riordan, J. F., and Coleman, J. E. (1963), *Proc. Nat. Acad. Sci. U. S. 49*, 937.
Weber, G., and Laurence, D. J. R. (1954), *Biochem. J. 56*, xxxi.

Fluorescence Polarization Studies of the Self-Association of Beef Liver Glutamate Dehydrogenase†

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ABSTRACT: Fluorescence polarization measurements on beef liver glutamate dehydrogenase conjugated with pyrenebutyric acid demonstrate that the association of this enzyme is adequately described by a reversible indefinite association with a single equilibrium constant. The data obtained with protein concentrations up to 0.7 mg/ml in 0.05 M potassium phosphate (pH 7.6) are consistent with a dissociation constant of 0.27 ± 0.02 mg/ml for an end-to-end association. The rotational relaxation time of the beef liver glutamate dehydrogenase monomer (320,000 g/mole) is 1030 ± 70 nsec at 20°. The retention of catalytic activity and sensitivity to effectors, ADP and GTP, shows that labeling causes little or no detectable change in the properties of the enzyme. Parallel experiments with dogfish glutamate dehydrogenase confirm the absence of significant association in this enzyme. Together these two cases demonstrate the usefulness of fluorescence polarization in the study of proteins undergoing self-association.

The indefinite self-association of proteins is of general interest because of its relevance to the self-assembly of biological structures and to the function of macromolecules.

Theoretical treatments of indefinite self-association with equal free energies of formation have been formulated both for ideal and nonideal cases (Van Holde and Rossetti, 1967; Adams and Lewis, 1968). Chun and Kim (1970) have presented a simplified method of graphical analysis for comparison of experimental data with the results predicted for monomer–*n*-mer and indefinite association equilibria.

Although several methods are available to obtain the thermodynamic parameters of association, sedimentation is most often applied (Roark and Yphantis, 1969; Cann, 1970) since it yields weight, number, and *z*-average molecular weights. Light scattering (Nichol *et al.*, 1964) and molecular-sieve chro-

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