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Structural Mapping of Nucleotide Binding Sites on Chloroplast Coupling Factor†

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ABSTRACT: Fluorescence resonance energy transfer was used to measure the distances between three nucleotide binding sites on solubilized chloroplast coupling factor from spinach and between each nucleotide site and two tyrosine residues which are important for catalytic activity. The nucleotide energy donor was 1,N^6-ethenoadenosine di- or triphosphate, and the nucleotide energy acceptor was 2′(3′)-(trinitrophenyl)adenosine diphosphate. The tyrosine residues were specifically labeled with 7-chloro-4-nitro-2,1,3-benzoxadiazole, which served as an energy acceptor. The results obtained indicate the three nucleotide binding sites form a triangle with sides of 44, 48, and 36 Å. (The assumption has been made in calculating these distances that the energy donor and acceptor rotate rapidly relative to the fluorescence lifetime.) Two of the nucleotide sites are approximately equidistant from each of the two tyrosines: one of the nucleotide sites is about 37 Å and the other about 41 Å from each tyrosine. The third nucleotide site is about 41 Å from one of the tyrosines and ≥41 Å from the other tyrosine.

The ATP synthetase complex from spinach chloroplasts consists of a soluble portion, chloroplast coupling factor 1 (CF_1),[1] containing five types of polypeptides (α, β, γ, δ, and ϵ) and a membrane component, CF_0, which may contain as many as four types of polypeptides (Pick & Racker, 1979). Nucleotide binding and catalysis occur on CF_1, while CF_0 serves to couple proton transport to phosphorylation. The molecular weight of CF_1 is 325 000 (Farron, 1970), and it has a probable polypeptide stoichiometry of $\alpha_2\beta_2\gamma\delta\epsilon_2$ (Baird & Hammes, 1976; Nelson, 1977; Binder et al., 1978). Isolated CF_1 cannot synthesize ATP; however, it is a Ca^{2+}-dependent ATPase when activated by various methods (Vambutas & Racker, 1965). This activity is abolished by reacting NBD-Cl with one tyrosine located on a β-polypeptide; a second tyrosine also can be modified (Deters et al., 1975).

Three nucleotide sites have been characterized on isolated CF_1 (Bruist & Hammes, 1981) and on the CF_1·CF_0 complex (Cerione & Hammes, 1981). These sites can be designated as follows: site 1 contains tightly bound ADP which cannot be dissociated by dialysis, but the ADP can be exchanged with medium nucleotides; site 2 binds MgATP very tightly; and site 3 binds nucleotides reversibly with typical dissociation constants in the micromolar range. Photoaffinity labeling studies suggest that the site binding MgATP tightly (site 2) is located primarily on the β-polypeptide and the site binding nucleotides reversibly (site 3) is near the interface between α- and β-polypeptides (Bruist & Hammes, 1981). The polypeptide location of the site binding ADP tightly (site 1) is not yet known. Sites 1 and 2 have been shown not to be catalytic sites, although they are probably of importance in regulation. The catalytic site is either site 3 or weak nucleotide binding sites that have not yet been characterized (Bruist & Hammes, 1981). In this work, the spatial relationships between the various nucleotide sites on isolated CF_1 have been investigated by fluorescence resonance energy transfer. The individual nucleotide sites were specifically labeled with ϵ-ADP and ϵ-ATP (energy donors) and TNP-ADP (energy acceptor), and the distances between the nucleotide sites, and between the nucleotide sites and the NBD-tyrosine sites, were measured.

Experimental Procedures

Materials. The ADP and ATP (vanadium free), ϵ-ADP, ϵ-ATP, DL-dithiothreitol, and β-mercaptoethanol were from Sigma Chemical Co. The concentrations of ADP and ATP were determined by assuming an extinction coefficient of 15 400 M^{-1} cm^{-1} at 259 nm, pH 7 (Beaven et al., 1955), and the concentrations of the ϵ-nucleotides were determined by using an extinction coefficient of 5600 M^{-1} cm^{-1} at 275 nm, pH 7 (Secrist et al., 1972). The NBD-Cl was from Pierce Chemical Co., and the TNP-ADP and TNP-ATP were from Molecular Probes. The concentrations of the TNP nucleotides were determined by assuming an extinction coefficient of 26 400 M^{-1} cm^{-1} at 408 nm and 18 500 M^{-1} cm^{-1} at 470 nm, pH 8 (Hiratsuka & Uchida, 1973). The [^{3}H]-ϵ-ADP was prepared as previously described (Secrist et al., 1972; Cantley & Hammes, 1975a) and was purified by thin-layer chromatography on cellulose sheets (Eastman) by using a solvent system of 1-propanol–NH_3–H_2O (6:3:1). The purification was

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[1] Abbreviations: CF_1, chloroplast coupling factor 1; ϵ-ADP or -ATP, 1,N^6-ethenoadenosine diphosphate or triphosphate; EDTA, ethylenediaminetetraacetic acid; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole; TNP-ADP or -ATP, 2′(3′)-(trinitrophenyl)adenosine di- or triphosphate.

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performed by applying the nucleotide samples 1.5 cm from the bottom of a thin-layer sheet and allowing the solvent to run to the top of the sheet (18 cm). The sheet was then dried, and the chromatography was repeated in the same solvent system. The R_f values for ϵ-ADP and ADP after the second chromatography were 0.21 and 0.14, respectively. All other chemicals used in these studies were the best available commercial grade, and all solutions were prepared with deionized distilled water.

Enzyme. The CF_1 was prepared from fresh spinach (Lien & Racker, 1971; Binder et al., 1978). Protein having a fluorescence ratio, 305 nm/340 nm (excitation at 280 nm), greater than 1.5 was collected and stored as an ammonium sulfate precipitate in 2 M ammonium sulfate, 10 mM Tris-HCl (pH 7.2), 1 mM EDTA, and 0.5 mM ATP. The molar concentration of CF_1 was obtained by using an extinction coefficient of 0.483 mL/(mg·cm) (Bruist & Hammes, 1981) and a molecular weight of 3.25×10^5 (Farron, 1970). When required, the latent enzyme was heat activated by heating at 60 °C for 4 min in 40 mM ATP, 30 mM Tris-HCl (pH 8.0), 7 mM dithiothreitol, and 2 mM EDTA (Farron & Racker, 1970).

Preparation of NBD-Tyr-CF_1 and Labeling of the Nucleotide Sites. NBD-modified CF_1, in which the NBD moiety is attached to one or two tyrosine groups per mole of enzyme (Deters et al., 1975; Cantley & Hammes, 1975a), was prepared by incubating CF_1 (5–16 μM) with NBD-Cl (40–70 μM) for ~60 min in 6 mM Mg^{2+}, 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 1 mM EDTA or in 40 mM Tris-HCl (pH 8.0) and 2 mM EDTA. The reaction of NBD with CF_1 was followed by monitoring the change in the absorbance at 400 nm in a Cary 118 C spectrophotometer thermostated at 23 °C. The 400-nm absorbances of an equal concentration of NBD-Cl in buffer (in the absence of protein) and of an equal concentration of CF_1 (in the absence of NBD-Cl) were subtracted from the 400-nm absorbance of the CF_1–NBD solution. The stoichiometry of NBD bound to CF_1 was determined by using an extinction coefficient of 10 700 M^{-1} cm^{-1} at 400 nm (Cantley & Hammes, 1975a).

The nucleotide site which binds ADP very tightly (nucleotide site 1) was labeled with TNP-ADP as follows: CF_1 (latent or heat activated) was passed through two consecutive 3-mL centrifuge columns (Penefsky, 1977) of Sephadex G50 fine equilibrated in 25 mM sucrose, 10 mM Tris-HCl (pH 8.0), and 2 mM EDTA and then incubated with TNP-ADP (150–200 μM) for 3–12 h at room temperature. For elimination of free or dissociable nucleotides, the incubation mixture (~0.5 mL) then was eluted through a Sephadex G25 medium column (1.2 cm i.d. × 50 cm) equilibrated in the sucrose–Tris–EDTA buffer, followed by an elution through a 3-mL Sephadex G50 fine column (equilibrated with the desired buffer), or the mixture was eluted through two consecutive 3-mL Sephadex G50 fine columns in the sucrose–Tris–EDTA buffer and then through a third G50 fine column equilibrated with the desired final buffer. The amount of TNP-ADP bound to CF_1 was determined by using extinction coefficients of 22 400 M^{-1} cm^{-1} at 408 nm and 25 100 M^{-1} cm^{-1} at 418 nm. These values were obtained by incubating 24 μM latent CF_1 with 1.4 μM TNP-ADP for 1 h in 6 mM Mg^{2+}, 50 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA and then determining the absorbances at 408 and 418 nm, after correction for the absorbance of CF_1 alone. It was assumed that under these conditions TNP-ADP binds reversibly at two equivalent sites on CF_1 with a dissociation constant of ~10 μM (see below), and the appropriate corrections were made for the small amount of unbound TNP-ADP in determining the extinction coefficients. The same values are obtained for ϵ_{408} and ϵ_{418} for TNP-ADP (1 μM) after an 18-h incubation with heat-activated CF_1 (7 μM), where it is assumed that all of the TNP-ADP is bound to the protein as a result of exchange with tightly bound ADP. The absorption spectra for TNP-ADP bound to CF_1 also are the same in different pH 8 buffers and in the presence and absence of metals.

In some cases, CF_1 (heat activated) was prelabeled with [^{3}H]ADP, prior to incubating the enzyme with TNP-ADP, in order to monitor the displacement of the tightly bound ADP. Prelabeling was carried out by incubating heat-activated CF_1 (30–40 μM) with [^{3}H]ADP (100–200 μM) for 4 h and then removing free and reversibly bound ADP by column chromatography. The amount of [^{3}H]ADP which is tightly bound to CF_1 was determined by measuring the radioactivity in 10 mL of ACS scintillation fluid.

The fluorescent adenine nucleotide analogue, ϵ-ADP, was exchanged with the tightly bound ADP on CF_1 by using procedures similar to those described for TNP-ADP: CF_1 (10–20 μM), separated from free and dissociable nucleotide, was incubated with 150–200 μM [^{3}H]-ϵ-ADP for 12 h in 25 mM sucrose, 10 mM Tris-HCl (pH 8.0), and 2 mM EDTA at room temperature, and then the incubation mixture was eluted through three consective 3-mL Sephadex G50 fine columns. The amount of ϵ-ADP tightly bound to CF_1 was determined by measuring the radioactivity in 10 mL of ACS scintillation fluid.

The nucleotide site which binds ATP or ϵ-ATP tightly in the presence of Mg^{2+} (nucleotide site 2; Carlier et al., 1979; Bruist & Hammes, 1981) was labeled by incubating CF_1 (10–35 μM) with [^{3}H]ATP (35–80 μM) or ϵ-ATP (60 μM) for 10–20 min in 6 mM Mg^{2+}, 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 1 mM EDTA at room temperature. Free and dissociable nucleotides were removed by eluting the incubation mixture through a Sephadex G25 column (1.2 cm i.d. × 20 cm) equilibrated in 25 mM sucrose, 10 mM Tris-HCl (pH 8.0), and 2 mM EDTA or through two consecutive 3-mL Sephadex G50 fine columns equilibrated with the incubation buffer. In some cases, the enzyme–ATP complex was concentrated by ammonium sulfate precipitation prior to column chromatography. The amount of [^{3}H]ATP tightly bound to CF_1 was determined by measuring the radioactivity in 10 mL of ACS scintillation fluid.

Specific labeling of the nucleotide site on CF_1 which binds all nucleotides reversibly in the presence and absence of metals (nucleotide site 3; Cantley & Hammes, 1975a; Bruist & Hammes, 1981) was achieved by prelabeling the site which binds ATP tightly (2) with [^{3}H]ATP or ϵ-ATP.

Fluorescence Measurements. Steady-state fluorescence measurements were made with a Hitachi Perkin-Elmer MPF-44B fluorescence spectrophotometer equipped with corrected spectrum and polarization accessories. The fluorometer was thermostated at 23 °C, unless otherwise indicated. The quantum yield for ϵ-ADP reversibly bound to CF_1, Q_b, was calculated by using the following relationship:

$$Q_b = [Q_f/(L_b)][(L_T)F_e/F_0 - (L_f)] \quad (1)$$

where Q_f is the quantum yield of ϵ-ADP free in solution (0.59, Cantley & Hammes, 1975b), (L_b), (L_f), and (L_T) are the concentrations of the bound ligand (ϵ-ADP), free ligand, and total ligand, respectively, and F_e and F_0 are the fluorescence emission (410-nm emission, 320-nm excitation) of ϵ-ADP in the presence and absence of enzyme, respectively. The determination of free and bound ϵ-ADP is described later. The quantum yield for ϵ-ADP bound at the tight ADP site (1) was

estimated by comparing the 410-nm emission (320-nm excitation) of the ϵ-nucleotide bound to CF_1 to the emission for an equal concentration of ϵ-ADP in buffer; the shapes of the emission spectra (excitation 320 nm) and the emission maxima are the same for ϵ-ADP tightly bound to CF_1 and free in solution. The concentration of tightly bound ϵ-ADP was determined by using [^{3}H]-ϵ-ADP. In all cases, the fluorescence emission of the ϵ-nucleotides, in the presence of enzyme, was corrected for light scattering of the protein by subtracting the apparent emission for an equal concentration of CF_1 (in the absence of nucleotide). The quantum yield for ϵ-ATP bound to nucleotide site 2 was assumed to be 0.425 (Carlier et al., 1979).

The steady-state fluorescence polarization, P, is defined

$$P = \frac{F_\parallel - F_\perp}{F_\parallel + F_\perp} \quad (2)$$

where $F_\parallel$ and $F_\perp$ are the fluorescence intensities when the emission polarizer is oriented in the parallel and perpendicular directions, respectively, relative to the excitation polarizer. The fluorescence polarization measurements for ϵ-ADP bound to CF_1 were made with the excitation at 320 nm (4-nm slit) and the emission at 410 nm (4-nm slit) with the application of a correction for the unequal transmission of horizontally and vertically polarized light by the emission monochromator grating (Chen & Bowman, 1965). Corrections also were made for the protein light scattering by measuring the apparent emission of an equal concentration of CF_1 in the absence of nucleotide.

Fluorescence resonance energy transfer measurements from ϵ-ADP and ϵ-ATP sites on CF_1 to the NBD-modified tyrosine residues on the β subunits were performed by adding ~5-fold excess of NBD-Cl over CF_1 and then simultaneously monitoring the change in absorbance at 400 nm and in fluorescence at 410 nm (excitation 320 nm). The fluorescence measurements were made intermittently after the first 5 min of the reaction to avoid possible photochemical decomposition. The reaction was allowed to proceed 60–70 min, and then 2 μL of 1.3 M β-mercaptoethanol solution was added to 200 μL of the reaction mixture to displace the NBD from the tyrosine residues. The observed fluorescence at 410 nm, after the addition of β-mercaptoethanol, represents the emission of the donor in the absence of bound acceptor. Small corrections were applied for inner filter effects of β-mercaptoethanol. These corrections were determined in control experiments where the fluorescence of the ϵ-nucleotides in buffer, in the presence and absence of β-mercaptoethanol, was measured. Estimates of the trivial energy transfer (absorption of the donor emission by NBD) indicated this was insignificant under the experimental conditions employed. The fluorescence of the ϵ-nucleotides bound to CF_1 always was corrected for the background fluorescence (or scattering) of the protein. Changes in this background due to the presence of NBD-Cl (inner filter effect) also were monitored, and the appropriate corrections were made. In experiments where TNP-ADP was used as an energy acceptor, corrections were applied for the decrease in the donor emission due to the absorption by TNP-ADP (trivial transfer). These corrections were obtained in control experiments where changes in the fluorescence of ϵ-ADP and ϵ-ATP in the presence of TNP-ADP (free in solution) were determined.

Results

Labeling of the Nucleotide Sites and NBD Modification. Each of the three nucleotide sites on CF_1 can be labeled specifically either with the fluorescent nucleotides ϵ-ADP and ϵ-ATP or with TNP-ADP or TNP-ATP. The nucleotide site which binds ADP tightly (site 1) was labeled with TNP-ADP and ϵ-ADP. Incubation of heat-activated CF_1 (4 μM), containing 0.84 mol of [^{3}H]ADP/mol of CF_1, with 140 μM TNP-ADP in 25 mM sucrose, 10 mM Tris-HCl (pH 8.0), and 2 mM EDTA results in a rapid uptake of the TNP-ADP (half-time ~5 min) and a corresponding displacement of the [^{3}H]ADP (Figure 1). The incomplete uptake of TNP-ADP at the tight ADP site (~0.6 mol of TNP-ADP/mol of CF_1) and the incomplete displacement of [^{3}H]ADP (~75% of the label displaced) could result from some of the heat-activated enzyme becoming inaccessible to exchange during the time period of the experiment, or from the establishment of an equilibrium between the competitive binding of ADP and TNP-ADP at site 1. The tightly bound ADP also can be exchanged with TNP-ATP, and the tight incorporation of TNP nucleotides also occcurs in the latent enzyme (0.7–1 mol of TNP-ADP/mol of CF_1 after a 3-h incubation). The ϵ-ADP also is capable of exchanging with tightly bound ADP on CF_1, but it is a much poorer substitute than the TNP nucleotides. Incubation of heat-activated CF_1 (20 μM) with ϵ-ADP (200 μM) for 12 h in 6 mM Mg^{2+}, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA, followed by elution of the incubation mixture through five consecutive 3-mL Sephadex G50 fine columns, results in only a small loss (~20%) of tightly bound [^{3}H]ADP. (The amount of [^{3}H]ADP remaining on the CF_1 sample incubated with ϵ-ADP is compared to the amount remaining on a control sample stored under identical conditions.) Similarly, 12-h incubations of latent CF_1 (15–20 μM) with [^{3}H]-ϵ-ADP (150–200 μM) result in a tight binding of only 0.26–0.29 mol of [^{3}H]-ϵ-ADP/mol of CF_1. The incorporation of ϵ-ADP is increased slightly (~1.3-fold), as judged by the emission at 410 nm (excitation 320 nm), when CF_1 is first incubated with ϵ-ADP for 7 h, separated from free and dissociable nucleotides by column chromatography, and then reincubated with ϵ-ADP for 12 h. This suggests that the low levels of incorporation reflect a weaker binding of ϵ-ADP, relative to ADP, at the site binding ADP tightly. The emission at 410 nm (excitation 320 nm) of ϵ-ADP is reduced 64% upon incorporation into CF_1 (quantum yield = 0.21), and the fluorescence polarization for the bound nucleotide is 0.10.

The nucleotide site which binds ATP tightly, in the presence of Mg^{2+} (site 2), was labeled with ϵ-ATP. CF_1 binds this fluorescent nucleotide tightly (Carlier et al., 1979), exactly

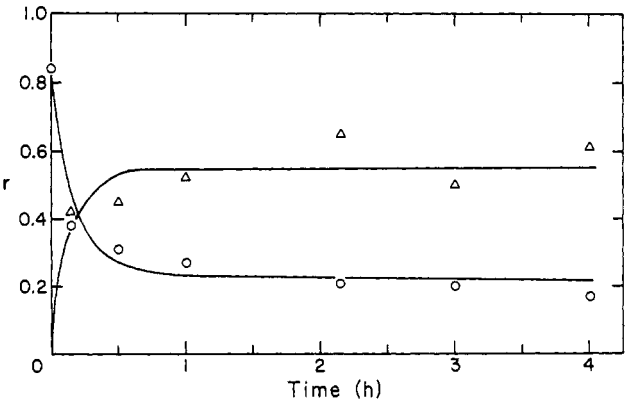

FIGURE 1: Plot of the moles of tightly bound [^{3}H]ADP (○) or TNP-ADP (△) per mole of CF_1, r, vs. the time of incubation with TNP-ADP. Tightly bound nucleotide was determined by measuring the radioactivity or the 408-nm absorbance, after eluting the incubation aliquot (0.5 mL) through three consecutive Sephadex G50 fine columns equilibrated in 25 mM sucrose, 10 mM Tris-HCl (pH 8.0), and 2 mM EDTA. The lines were calculated by assuming a first-order reaction.

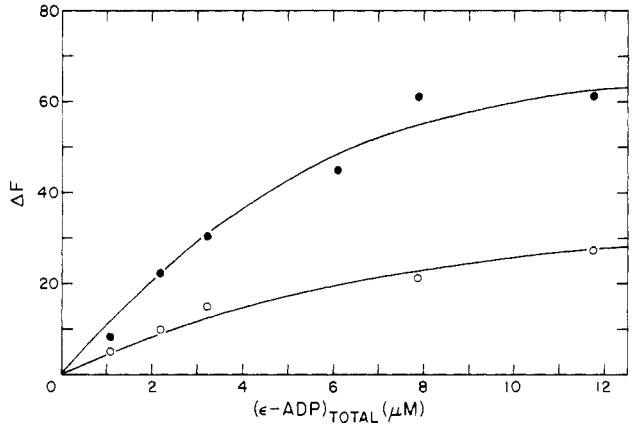

FIGURE 2: Typical plots of the change in the fluorescence emission of ε-ADP, ΔF (arbitrary units), upon binding to latent CF_1 (○) or CF_1·TNP-ADP (●) vs. the total concentration of ε-ADP. The CF_1 contained 1.2 mol of [^{3}H]ATP/mol of CF_1 and was equilibrated with ε-ADP for 30 min at room temperature in 6 mM Mg^{2+}, 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 1 mM EDTA prior to making a fluorescence measurement (emission 410 nm, excitation 320 nm).

as is the case with ATP (Bruist & Hammes, 1981). The tight incorporation of ε-ATP (1:1) results in 20–30% quenching of the fluorescence emission of this nucleotide at 410 nm (320-nm excitation). (The amount of quenching varies within this range for different enzyme preparations and for the heat-activated and latent enzyme.) The fluorescence polarization for ε-ATP bound to nucleotide site 2 is 0.23 (Carlier et al., 1979). The nucleotide site which binds all nucleotides reversibly (site 3) has been labeled with ε-ADP and TNP-ADP. Since in the absence of Mg^{2+}, nucleotide sites 2 and 3 appear to bind all nucleotides with essentially equal affinity (Cantley & Hammes, 1975a; Bruist & Hammes, 1981), specific labeling at site 3 was ensured by prelabeling site 2 with [^{3}H]ATP (or with ε-ATP when TNP-ADP was the acceptor at site 3). The stoichiometries determined for [^{3}H]ATP labeling of the Mg^{2+}-dependent tight ATP site varied from 1.0 to 1.3 mol of [^{3}H]ATP/mol of CF_1. The amount of [^{3}H]ATP bound in excess of 1 mol of ATP/mol of CF_1 may reflect either some exchange into the tight ADP site or an error in the molecular weight of CF_1.

The binding of ε-ADP to CF_1, prelabeled with Mg[^{3}H]ATP, was monitored by following changes in the fluorescence emission of the ε-nucleotide. Some typical plots of the change in fluorescence emission vs. ε-ADP concentration (total) are presented in Figure 2. The data were fit by regression analysis to

$$\Delta F/\Delta F_{max} = (L_b)/(E_0) \quad (3)$$

$$2(L_b) = (E_0) + K + (L_T) - \{[(E_0) + K + (L_T)]^2 - 4(E_0)(L_T)\}^{1/2} \quad (4)$$

Here, ΔF is the observed fluorescence for a given concentration of ε-ADP in the presence of CF_1 subtracted from the fluorescence of ε-ADP in buffer alone, ΔF_{max} is the maximum change in fluorescence which occurs upon saturation of the nucleotide binding site, (L_b) is the concentration of bound ε-ADP, (E_0) is the total concentration of CF_1, K is the dissociation constant for ε-ADP binding to CF_1, and (L_T) is the total concentration of the fluorescent nucleotide. (Equation 4 is derived from the concentration relationship specified by the dissociation constant and the mass conservation relationships for enzyme and ligand.) The dissociation constant for ε-ADP, obtained from titration studies, was 1.87 (±0.78) μM (average of two studies). This value is in good agreement with the dissociation constant (1.8 μM) determined for ε-ADP binding to a CF_1 complex which does not contain tightly bound ATP at nucleotide site 2 (Cantley & Hammes, 1975a), supporting the suggestion that prelabeling CF_1 with ATP (at site 2) has no effect on the reversible binding of nucleotides to site 3 (Bruist & Hammes, 1981). The incorporation of TNP-ADP into the site binding ADP tightly also appears to have no effect on the reversible binding by nucleotide to site 3 (K = 1.66 μM). The quenching in the fluorescence emission at 410 nm, determined for ε-ADP binding to nucleotide site 3, was somewhat variable, ranging from 13 to 37%. This corresponds to a range of 0.38–0.51 for the quantum yield of ε-ADP bound to nucleotide site 3. In order to eliminate errors in the distance measurements due to these variations in quantum yield, the quantum yield for ε-ADP bound to the dissociable nucleotide site was determined (by using eq 1 and assuming the dissociation constant for ε-ADP binding to CF_1 is 2 μM) immediately prior to performing energy transfer experiments involving this site.

The polarization of ε-ADP, bound at nucleotide site 3, was calculated from eq 5 where it is assumed that the polarization of the fluorescence of the ligand free in solution (0.002) is small

$$1/P = 1/P_b + \frac{(F_\parallel + F_\perp)_f}{(F_\parallel + F_\perp)_b P_b}[(L_f)/(L_b)] \quad (5)$$

compared with the polarization of the ligand bound to the enzyme (Cantley & Hammes, 1975b). In this equation, P is the measured polarization, P_b is the polarization of the bound ligand, $(F_\parallel + F_\perp)_f$ and $(F_\parallel + F_\perp)_b$ are the sums of the parallel and perpendicular components of the fluorescence of the ligand free in solution and bound to the protein, respectively, and (L_f) and (L_b) are the concentrations of the ligand free in solution and bound to the protein, respectively. The latter value was determined by using eq 1 and assuming K = 2 μM. A plot of $1/P$ vs. $(L_f)/(L_b)$ is linear (data not shown) and yields a value for P_b of 0.25, similar to the value reported earlier for the reversible binding of ε-nucleotides to sites 2 and 3 of CF_1 (Cantley & Hammes, 1975b).

Preliminary measurements of the reversible binding of TNP-ADP to CF_1 using forced dialysis indicate binding stoichiometries similar to those found with ADP (Bruist & Hammes, 1981).

CF_1 also was labeled with NBD-Cl, at one or two sites per enzyme complex. The modification of a single tyrosine residue eliminates essentially all the catalytic activity. The modification of a second tyrosine residue proceeds at about one-tenth the rate of the modification of the first tyrosine residue.

Measurements of Energy Transfer between Nucleotide Sites. The distances separating the various nucleotide sites on CF_1 were determined from measurements of resonance energy transfer (Förster, 1959, 1965). The efficiency of energy transfer, E, written in terms of the quantum yield of an energy donor in the presence, Q_{DA}, and absence, Q_D, of an acceptor is

$$E = 1 - Q_{DA}/Q_D \quad (6)$$

The distance between the donor and acceptor sites, R, for the case of one donor and one acceptor, is related to the energy transfer efficiency by

$$R = R_0(E^{-1} - 1)^{1/6} \quad (7)$$

where R_0 is the distance at which E is 50%. The value of R_0 is given by

$$R_0 = (9.79 \times 10^3)(JK^2Q_Dn^{-4})^{1/6}\ \text{Å} \quad (8)$$

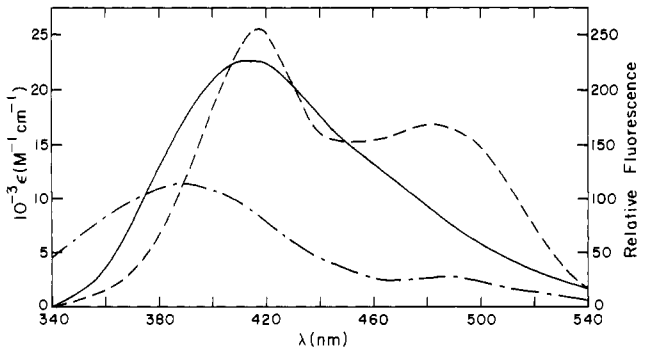

FIGURE 3: Overlap of the corrected fluorescence emission of ϵ-ADP excited at 320 nm (—) with the difference extinction coefficient (ϵ) of NBD-Tyr (·····) and with the difference extinction coefficient (ϵ) of CF_1·TNP-ADP (---) in 50 mM NaCl, 10 mM Tris-HCl (pH 8.0), 6 mM Mg^{2+}, and 1 mM EDTA.

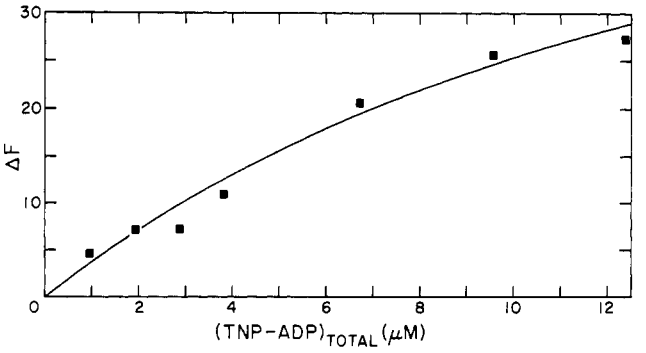

FIGURE 4: Plot of the percent quenching in the fluorescence emission (410 nm, excitation 320 nm) of ϵ-ATP, ΔF, upon the binding of TNP-ADP to latent CF_1. The titration was performed in 50 mM NaCl, 10 mM Tris-HCl (pH 8.0), 6 mM Mg^{2+}, and 1 mM EDTA at 23 °C. TNP-ADP, at each concentration, was allowed to equilibrate with the CF_1·ϵ-ATP complex (3 μM) 5 min prior to measuring the emission at 410 nm.

where n is the refractive index of the medium, Q_D is the quantum yield of the donor in the absence of the acceptor, K^2 is an orientation factor characterizing the relative orientation of the donor and acceptor transition dipoles, and J is the spectral overlap integral of the donor fluorescence and acceptor absorption spectra. The overlap integrals for the various donor–acceptor pairs were calculated as previously described (Cantley & Hammes, 1975b), by using the spectral data shown in Figure 3. In the calculations of R_0, K^2 was assumed to have a value of $^2/_3$ which represents the case where the donor and acceptor rotate rapidly relative to the donor fluorescence lifetime; the validity of this assumption is considered later. The value of n was assumed to be 1.4, i.e., the refractive index of water.

The energy transfer efficiency between nucleotide site 1 and nucleotide site 3 was determined by using TNP-ADP as an energy acceptor at nucleotide site 1 (0.77 mol of TNP-ADP/mol of CF_1) and ϵ-ADP as an energy donor at nucleotide site 3. Nucleotide site 2 in both native and TNP-ADP-labeled CF_1 was prelabeled with ATP. The quantum yields for ϵ-ADP bound to nucleotide site 1 on both of these (latent) enzyme complexes were determined from titration studies by following the changes in the fluorescence of ϵ-ADP (Figure 1). In these experiments, the quantum yield for ϵ-ADP reversibly bound to CF_1 in 6 mM Mg^{2+}, 50 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA was determined to be 0.514, and the quantum yield for ϵ-ADP bound to the dissociable nucleotide site on CF_1·TNP-ADP was 0.415. Therefore, the efficiency of energy transfer is 0.21 (0.25/mol of acceptor). The energy transfer efficiency between nucleotide site 1 and nucleotide site 3 in the heat-activated enzyme could not be determined from titration experiments because the change in the emission of ϵ-ADP associated with binding to the enzyme in the absence of the acceptor was very small (<5%). A comparison of the fluoroscence emission for ϵ-ADP free in solution and bound at nucleotide site 3 of heat-activated CF_1 and CF_1·TNP-ADP complexes was made over a nucleotide concentration range where [ϵ-ADP] ≤ [CF_1]. An average energy transfer efficiency of 0.23 ± 0.09 (0.26/mol of acceptor) was determined (four ϵ-ADP concentrations) by assuming a value of 2 μM for the dissociation constant for ϵ-ADP.

The energy transfer efficiency between nucleotide site 1 and nucleotide site 2 on latent CF_1 was determined by using TNP-ADP as an energy acceptor at nucleotide site 1 (0.7 mol of TNP-ADP/mol of CF_1) and ϵ-ATP as an energy donor at site 2. Since the donor is bound very tightly to the enzyme, no correction is necessary for the fluorescence due to nucleotide which is free in solution. The ratios for the 410-nm emission of ϵ-ATP bound to CF_1 (5 μM) and to CF_1·TNP-ADP (5 μM) were determined at four nucleotide concentrations ranging from 1.1 to 5.1 μM ϵ-ATP. The average energy transfer efficiency was 0.22 ± 0.01 (0.31/mol of acceptor). A similar energy transfer efficiency (0.37/mol of acceptor) was calculated from an experiment where a stoichiometric amount of ϵ-ATP was added to heat-activated CF_1 and CF_1·TNP-ADP, and then the ratio of the emission for ϵ-ATP bound to both complexes was determined after 10 min. A steady decline in the energy transfer efficiency was observed as repeated measurements were taken with the heat-activated enzyme (the efficiency was ~0.25/mol of acceptor 1 h after the initial measurement), suggesting a slow dissociation of the acceptor and/or donor.

The quenching of the fluorescence of ϵ-ATP bound to site 2, upon the reversible binding of TNP-ADP to site 3, was used to determine the energy transfer efficiency between these two sites. The change in fluorescence of the latent CF_1·ϵ-ATP complex (410-nm emission and 320-nm excitation) accompanying titration of the complex with TNP-ADP is shown in Figure 4. The solid line was obtained by fitting the data to eq 3 and 4, assuming one accessible binding site. The best fit yielded a dissociation constant for TNP-ADP of 12.8 μM and a final level of quenching of 63%. In order to minimize the possibility of exchange between TNP-ADP and tightly bound ADP (at site 1), the latent enzyme was used in these studies, and the experiment was completed in ~30 min. The highest total TNP-ADP concentration examined was less than 13 μM to avoid weak or nonspecific binding of TNP-ADP. Essentially 100% of the ϵ-ATP emission is regained immediately upon the addition of excess ADP (100 μM), after correction for inner filter effects and trivial transfer to TNP-ADP, indicating that the energy transfer is the result of TNP-ADP binding at the dissociable nucleotide site. Table I summarizes the distances between the various nucleotide sites calculated with eq 7.

Measurements of Energy Transfer between the Nucleotide Sites and the NBD-Tyr Sites. The efficiencies of energy transfer between the nucleotide sites and the NBD-Tyr sites were estimated by monitoring the donor (ϵ-ATP or ϵ-ADP) fluorescence during the time course of the modification of latent CF_1 with NBD-Cl. Heat-activated CF_1 was not used in these studies because NBD-Cl also reacts with sulfhydryl groups on the heat-activated enzyme (Cantley & Hammes, 1975a). Plots of the efficiency of energy transfer vs. the moles of NBD bound per mole of CF_1, for the cases where nucleotide site 2 is occupied with ϵ-ATP and where nucleotide site 3 is occupied with ϵ-ADP, are shown in Figure 5. The data were

Table I: Energy Transfer Parameters

donor	location[a]	acceptor	location[a]	R_0 (Å)	E[b]	R (Å)
ϵ-ATP	2	TNP-ADP	1	39.1	0.31	45
		TNP-ADP[c]	1	39.1	0.37	43
		TNP-ADP	3	39.1	0.63	36
ϵ-ADP	3	TNP-ADP	1	40.3	0.25	48
		TNP-ADP[c]	1	41.2	0.26	48
ϵ-ATP	2	NBD	A	33.6	0.38 (0.37)[d]	36 (37)[d]
		NBD	B			42 (37)[d]
ϵ-ADP	3	NBD	A	33.0	0.22	41 (41)[d]
		NBD	B			41 (41)[d]
ϵ-ADP	1	NBD	A	29.9	0.14	41
		NBD	B			(≥41)

[a] The numbers designate the nucleotide binding sites; A is the first tyrosine modified by NBD, and B is the second tyrosine modified. [b] Energy transfer efficiency per mole of acceptor. [c] Heat-activated CF_1. [d] The values in parentheses were obtained by fitting the data to eq 10, and the other values were obtained by fitting the data to eq 9.

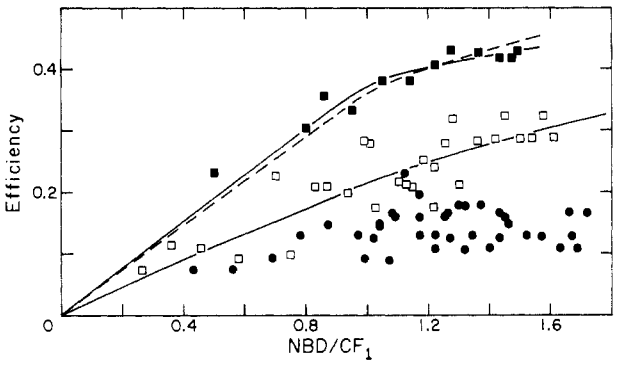

FIGURE 5: Plot of the efficiency of energy transfer from ϵ-ATP at the site binding MgATP tightly (2) (■) or ϵ-ADP at the site binding nucleotides reversibly (3) (□) and from ϵ-ADP at the site binding ADP tightly (1) (●) vs. the number of moles of NBD bound to tyrosine residues on CF_1. The upper solid line is the best fit to eq 9; the dashed line is the best fit to eq 10, and the lower solid line is the best fit to both eq 9 and 10.

fit to two models: one assumes that the tyrosines are not equivalent prior to modification (eq 9), and one assumes that

$$E = \frac{n_1(R_0/R_1)^6}{1 + (R_0/R_1)^6} + \frac{n_2[(R_0/R_1)^6 + (R_0/R_2)^6]}{[1 + (R_0/R_1)^6 + (R_0/R_2)^6]} \quad (9)$$

the two tyrosines have identical reactivity before reaction with NBD, with the second tyrosine becoming less reactive after modification of the first tyrosine (eq 10) [cf. Hammes (1981)

$$E = \frac{n_3(R_0/R_1)^6}{1 + (R_0/R_1)^6} + \frac{n_3(R_0/R_2)^6}{1 + (R_0/R_2)^6} + \frac{n_2[(R_0/R_1)^6 + (R_0/R_2)^6]}{[1 + (R_0/R_1)^6 + (R_0/R_2)^6]} \quad (10)$$

for calculation of E when multiple energy donors and acceptors are present]. In eq 9 and 10, n_1 and n_2 represent the total fraction of enzyme containing 1 and 2 mol of NBD/mol of CF_1, respectively, n_3 represents the fraction of enzyme containing 1 mol of NBD/mol of CF_1 for each of the two equivalent adducts formed, and the distances to the two tyrosine residues are R_1 and R_2. Since the first NBD-Tyr adduct forms at a much faster rate (~10-fold) than the second, the values for n_1, n_2, and n_3 were calculated by assuming that all CF_1 molecules contained one NBD before a second NBD was added.

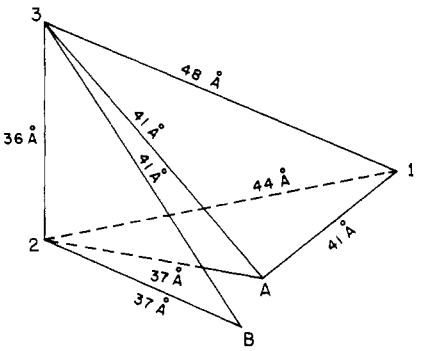

FIGURE 6: Model for the spatial relationships of the three nucleotide sites and the two NBD-Tyr sites. 1 represents the site binding ADP tightly, 2 the site binding MgATP tightly, and 3 the site binding nucleotides reversibly; A is the Tyr modified first with NBD, and B is the site modified second. The distances between nucleotide sites 2 and 3 and the NBD-Tyr residues are those obtained from the best fits to eq 10.

The best fits to eq 9 and 10, for the case where ϵ-ATP is bound to nucleotide site 2, are represented by the upper solid and dashed lines, respectively, in Figure 5 and yield an energy transfer efficiency of ~0.37 for ~1 mol of NBD/mol of CF_1. This is similar to the value (0.43) previously reported (Carlier et al., 1979). The values obtained for the distances between the two NBD groups and the site binding ATP tightly are given in Table I. When ϵ-ADP is the energy donor at nucleotide site 3, essentially identical fits are obtained with eq 9 and 10 (the solid line in Figure 5), and the distances between the two NBD-Tyr sites and nucleotide site 3 are identical (Table I). In these determinations, the energy transfer efficiencies were corrected for the fluorescence emission which is due to unbound ϵ-ADP, assuming a dissociation constant of 2 μM for the reversible binding of ϵ-ADP. An 8–12-fold excess of enzyme over ϵ-ADP was used to minimize the significance of this correction. The average energy transfer efficiency for ϵ-ADP bound to CF_1 molecules (0.86–1.27 mol of NBD/mol of CF_1), which have both nucleotide sites 2 and 3 accessible, is 0.23/mol of acceptor, assuming a dissociation constant for ϵ-ADP of 2 μM. A similar efficiency (0.17/mol of acceptor) was reported from studies where the amounts of free and bound ϵ-ADP were determined directly (Cantley & Hammes, 1975b).

Figure 5 also shows a plot of the energy transfer efficiency vs. the amount of NBD/CF_1, for the case where ϵ-ADP is bound at nucleotide site 1. The energy transfer efficiency between the most reactive tyrosine residue and the tight ADP site is ~0.14, yielding a distance which is similar to those separating the NBD sites from the other nucleotide sites (Table I). The best fits to eq 9 and 10 suggest that the second tyrosine modified by NBD contributes little, or not at all, to the energy transfer. However, the sensitivity of the fitting procedure to small variations in the energy transfer efficiency when the efficiency is low does not permit a reliable conclusion to be reached with regard to this point.

Discussion

In this work, the distances between three distinct nucleotide sites on CF_1, and between these nucleotide sites and two NBD-modified tyrosines, have been determined. The results are summarized by the model in Figure 6; the distance between

the two NBD sites has not yet been determined. Two major assumptions were made in calculating these distances: (1) the donor fluorescence quenching is due to energy transfer to the acceptor and not to changes in the environment of the donor induced by the binding of acceptor, and (2) the donor and acceptor rotate rapidly relative to the donor fluorescence lifetime so that $K^2 \simeq {}^2/_3$.

Although acceptor-induced environmental changes cannot be unequivocally excluded, several observations suggest they are not of importance. The following evidence suggests acceptor-induced conformational changes are not causing the quenching of fluorescence for cases where TNP-ADP is the energy acceptor at nucleotide sites 1 and 3, respectively. (1) The binding affinities of CF_1 for ϵ-ADP at site 3, and ϵ-ATP at site 2, are not significantly altered by the presence of TNP-ADP at site 1. (2) The fluorescence of ϵ-ATP, bound at site 2, is not affected when ADP, instead of TNP-ADP, binds at site 3. The situation is not as clear when NBD is the acceptor. Neither the binding of ϵ-ADP to sites 2 and 3 nor the tight binding of MgATP at site 2 appears to be significantly altered by the NBD modification, under the conditions these experiments were performed. Also, significant dissociation of ϵ-ADP from site 1 during the NBD modification does not appear to occur. However, none of these results excludes small conformational changes that might influence the energy transfer measurements. In fact, NBD has been shown to induce conformational changes in CF_1 (Holowka & Hammes, 1977).

The assumption that $K^2 = {}^2/_3$ has been discussed often [cf. Stryer (1978) and Dale et al. (1979)]. The upper and lower bounds for K^2 can be calculated from the fluorescence polarizations of the energy donor (Dale et al., 1979). The fluorescence polarizations of ϵ-ADP bound at nucleotide sites 1 and 3 are 0.10 and 0.25, respectively, and that of ϵ-ATP bound at nucleotide site 2 is 0.23 (Carlier et al., 1979). These give a *maximum* range for the distances between nucleotide sites and between nucleotide sites 2 and 3 and NBD-tyrosine of about ±20%. The *maximum* range in the distance between nucleotide site 1 and NBD-tyrosine is about ±15%. In the calculation of these ranges, the assumption was made that the energy acceptor has no rotational freedom [Figure 9 of Dale et al. (1979)]. The more realistic inclusion of acceptor rotational freedom would reduce the ranges, but unfortunately, the quantum yields of the acceptors are insufficient to permit determination of their polarizations. A realistic assessment of the probable uncertainty in the measured distances due to the uncertainty in K^2 is about ±10%.

The quenching of ϵ-ADP fluorescence upon binding to nucleotide site 1 is more than 2-fold greater than the quenching accompanying binding to the other two nucleotide sites. This suggests a very nonpolar environment (Secrist et al., 1972). In contrast, the fluorescence polarization of ϵ-ADP at site 1 is considerably less than that at the other two sites, indicating more rotational freedom of the adenine ring system at site 1. This may be related to the weaker binding of ϵ-ADP to site 1 compared with ADP and TNP-ADP.

The results obtained indicate that the three nucleotide sites on CF_1 are not very close to one another. However, the exchange of [^{3}H]ADP into site 1 requires the binding of nucleotides at another site, and the exchange is greatly enhanced during ATP hydrolysis (Bruist & Hammes, 1981). Therefore, conformational coupling between the nucleotide sites must occur. The distances between nucleotide sites 1 and 3 or between sites 1 and 2 are not altered by heat activation of the enzyme.

The nucleotide sites are not close to the tyrosines modified with NBD-Cl. The catalytic site is either nucleotide site 3 or a site not yet characterized that binds nucleotides weakly (Bruist & Hammes, 1981). In the former case, inactivation of the enzyme by NBD modification must be due to conformational changes. The two NBD sites are equidistant from nucleotide site 3 and almost equidistant from site 2. The small difference in the latter case, which is obtained when fitting the data to a model by assuming a preexisting difference in the reactivities of the two tyrosine residues, could be due to a slight dissociation of ϵ-ATP from the tight ATP site or to a small conformational change. These results suggest either that the two NBD sites are close to each other or that they are symmetrically placed with respect to nucleotide sites 2 and 3. The possibility of a small conformational change accompanying nucleotide binding to site 2 can be inferred by the finding that the energy transfer efficiency from ϵ-ATP at site 2 to the first tyrosine modified by NBD is 0.37 and that from ϵ-ADP at site 3 to the same tyrosine is 0.22 with site 2 occupied by ATP, while that from ϵ-ADP at *both* sites 2 and 3 is 0.23. If this nonadditivity of efficiencies is due to a conformational change, the change in distance between the nucleotide site and the NBD-modified tyrosine can be estimated to be a few angstroms. The amount of energy transfer between ϵ-ADP at nucleotide site 1 and NBD-tyrosine is quite small; as a result, the placement of nucleotide site 1 with respect to the two NBD-tyrosines cannot be unambiguously ascertained.

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Primary Structures of Two Low Molecular Weight Proteinase Inhibitors from Potatoes†

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ABSTRACT: The amino acid sequences of two low molecular weight proteinase inhibitors from Russet Burbank potatoes have been determined. One of these, a chymotrypsin inhibitor, is a peptide of 52 amino acid residues, while the second inhibitor, which is specific for trypsin, contains 51 amino acid residues. These peptides are highly homologous, differing at only nine positions. At position 38, the chymotrypsin inhibitor possesses leucine and the trypsin inhibitor an arginine. This difference probably represents the P_1 sites, which are consistent with the respective specificities of the two inhibitors. The inhibitors are also homologous with potato inhibitor II and with an inhibitor previously isolated from eggplants.

Russet Burbank potato tubers have been shown to contain a variety of heat-stable proteinase inhibitors. In addition to inhibitor I (M_r 40 000; Melville & Ryan, 1972) and inhibitor II (M_r 19 500; Bryant et al., 1976; Iwasaki et al., 1972), several low molecular weight inhibitors have been detected. The latter group contains a carboxypeptidase inhibitor, which has been extensively characterized (Rancour & Ryan, 1968; Hass & Ryan, 1981), and at least three inhibitors of serine proteinases (Pearce et al., 1981). This report describes the determination of the amino acid sequences of two of the polypeptide serine proteinase inhibitors, a chymotrypsin inhibitor I (PCI-I)[1] and a trypsin inhibitor (PTI). PCI-I is a potent inhibitor of chymotrypsin with little effect on trypsin (Hass et al., 1976), and, conversely, PTI is specific only for trypsin (Pearce et al., 1981). The three polypeptide serine proteinase inhibitors are highly homologous, since they all exhibit strong immunological cross-reactivity against rabbit anti-PCI-I serum (Pearce et al., 1981).

Experimental Procedures

Materials. The polypeptide chymotrypsin inhibitor (PCI-I) and the polypeptide trypsin inhibitor (PTI) were prepared as described by Hass et al. (1976) and by Pearce et al. (1981), respectively. The *S*-Cm derivatives were prepared by reduction of the inhibitors with 0.1 M 2-mercaptoethanol in 6 M guanidine hydrochloride and 0.1 M Tris-HCl (pH 8.0) followed by treatment with a 1.5-fold M excess of bromo[1-^{14}C]acetate (Crestfield et al., 1963).

Trypsin treated with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone, chymotrypsin, and carboxypeptidase B were purchased from Sigma Chemical Co. Pyridine was redistilled from ninhydrin prior to use in chromotographic systems (Hill & Delaney, 1967), and all other chemicals were reagent grade or better and were used without further purification.

Analytical Procedures. Amino acid analyses were performed according to Spackman et al. (1958) on samples which had been hydrolyzed for 18 h in 6 N HCl at 110 °C (Moore & Stein, 1963). Peptide purity was assessed by high-voltage paper electrophoresis at pH 3.6 or 6.5 (Bennett, 1967). Peptides were routinely detected on dried chromatograms by using fluorescamine (Mendez & Lai, 1975).

Automatic Edman Degradation. The amino acid sequences were determined with a Beckman 890C sequencer according to the procedures of Hermodson et al. (1972) with the exceptions that Polybrene (3 mg) was added to the sequencer cup with the peptides (100–200 nmol) and in the case of the whole protein 0.1 M Quadrol buffer (Pierce Chemical Co.) was used. The program used was identical with that used by Hermodson et al. (1977) except that a 10-min extraction with ethyl acetate was performed immediately following the benzene-extraction step. Pth-amino acids were identified by high-pressure liquid chromatography employing a slight modification of the procedures of Zimmerman et al. (1977). No identifications were made unless the peak to background ratio was at least 3.

Cleavage at the Asn–Gly Bond. The *S*-Cm derivative of each protein (800 nmol) was cleaved at the Asn_{19}–Gly_{20} bond (Bornstein & Balian, 1970). Hydroxylamine hydrochloride (2.8 g) and guanidine hydrochloride (6 g) were dissolved in enough water to make 15 mL of solution. The solution was titrated to pH 7 with 12 N NaOH while stirring in an ice bath and then adjusted to 20 mL with water. Three milliliters of this solution was added to the protein, the pH was adjusted

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[1] Abbreviations: *S*-Cm, *S*-carboxymethyl; PCI-I, polypeptide chymotrypsin inhibitor I; PTI, polypeptide trypsin inhibitor; Pth, phenylthiohydantoin; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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