Ohlsson, P.-I., Paul, K.-G., & Wold, S. (1984) *Acta Chem. Scand., Ser. B B38*, 853–859.
Pruitt, K. M., & Reiter, B. (1985) in *The Lactoperoxidase System: Chemistry and Biological Significance* (Pruitt, K. M., & Tenovuo, J., Eds.) pp 143–178, Marcel Dekker, New York.
Pruitt, K. M., Mansson-Rahemtulla, B., & Tenovuo, J. (1983) *Arch. Oral Biol. 28*, 517–525.
Pruitt, K. M., Tenovuo, J., Mansson-Rahemtulla, B., Harrington, P. G., & Baldone, D. C. (1986) *Biochim. Biophys. Acta 870*, 385–391.
Ralston, M. (1985) in *BMDP Statistical Software Manual* (Dixon, W. J., Ed.) pp 305–314, University of California, Berkeley, CA.
Shiro, Y., & Morishima, I. (1986) *Biochemistry 25*, 5844–5849.
Sievers, G. (1985) *Protides Biol. Fluids 32*, 129–132.
Tenovuo, J., & Anttonen, T. (1980) *Caries Res. 14*, 269–275.
Tenovuo, J., & Pruitt, K. M. (1984) *J. Oral Pathol. 13*, 574–584.
Thomas, E. L. (1985a) in *The Lactoperoxidase System: Chemistry and Biological Significance* (Pruitt, K. M., & Tenovuo, J., Eds.) pp 31–53, Marcel Dekker, New York.
Thomas, E. L. (1985b) in *The Lactoperoxidase System: Chemistry and Biological Significance* (Pruitt, K. M., & Tenovuo, J., Eds.) pp 179–202, Marcel Dekker, New York.
Wever, R., Kast, W. M., Kasinoedin, J. H., & Boelens, R. (1982) *Biochim. Biophys. Acta 709*, 212–219.

Characterization of Three-Subunit Chloroplast Coupling Factor†

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ABSTRACT: The δ- and ε-polypeptides were removed from chloroplast coupling factor 1 (CF_1). The resulting enzyme, $CF_1(-\delta,\epsilon)$, is a stable active ATPase containing only α-, β-, and γ-polypeptides. The dependence of the steady-state kinetics of ATP hydrolysis catalyzed by $CF_1(-\delta,\epsilon)$ on the concentrations of ATP and ADP was found to be essentially the same as by activated CF_1. Nucleotide binding studies with $CF_1(-\delta,\epsilon)$ revealed three binding sites: a nondissociable ADP site (site 1), a tight MgATP binding site (site 2), and a site that binds ADP and ATP with a dissociation constant in the micromolar range (site 3). Similar results have been obtained with CF_1. For both CF_1 and $CF_1(-\delta,\epsilon)$, the binding of MgATP at site 2 is tight only in the presence of Mg^{2+}. Fluorescence resonance energy transfer was used to map distances between the γ-sulfhydryl ("dark" site) and γ-disulfide and between the γ-sulfhydryl and the three nucleotide sites. These distances are within 5% of the corresponding distances on CF_1. These results indicate that removal of the δ- and ε-polypeptides from CF_1 does not cause significant changes in the structure, kinetics, and nucleotide binding sites of the enzyme.

The spinach chloroplast ATP synthase catalyzes the photophosphorylation of ADP. The enzyme has two distinct parts: a membrane-imbedded portion, CF_0, and an extrinsic portion, CF_1.[1] This latter part can be readily stripped from the thylakoid membranes (Lien & Racker, 1971) and isolated as a soluble complex. CF_1 contains the substrate binding sites and is a latent ATPase. It contains five different polypeptide chains, α, β, γ, δ, and ε, with a probable stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$ (Moroney et al., 1983).

The structure of CF_1 has been extensively investigated with fluorescent methods (Cerione & Hammes, 1982; Cerione et al., 1983; Snyder & Hammes, 1984, 1985; Nalin et al., 1985; Richter et al., 1985; Schinkel & Hammes, 1986; McCarty & Hammes, 1987). Studies of nucleotide binding sites have revealed the existence of a site that binds ADP tightly (site 1), a site that binds MgATP tightly (site 2), and a site that binds ADP and ATP with a dissociation constant in the micromolar range (site 3) (Bruist & Hammes, 1981, 1982). The α- and β-polypeptides contain the nucleotide binding sites (Bruist & Hammes, 1982; Czarnecki et al., 1982; Bar-Zvi & Shavit, 1982; Kambouris & Hammes, 1985; Admon & Hammes, 1987). The γ-polypeptide is involved in regulation of catalysis and probably in proton translocation (McCarty & Moroney, 1985). The δ-polypeptide has no effect on the ATPase activity of CF_1, but it makes an important contribution to the asymmetric shape of CF_1 (Schinkel & Hammes, 1986) and to the efficient coupling of catalysis and proton pumping. The ε-polypeptide is an ATPase inhibitor (Richter et al., 1985).

A method has been described for preparing δ- and ε-deficient CF_1 (Richter et al., 1984). This enzyme is an active ATPase. In the present work, $CF_1(-\delta,\epsilon)$ was studied with respect to its nucleotide binding sites, its steady-state kinetics, and its structure.

Materials and Methods

Chemicals. ADP and ATP (vanadium free) were from Sigma Chemical Co.; [^{3}H]ATP was from New England Nuclear and [γ-^{32}P]ATP from Amersham Corp. ATP was purified as previously described (Bruist & Hammes, 1981). CPM, FM, and TNP-ATP were from Molecular Probes, Inc.,

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[1] Abbreviations: CF_1, chloroplast coupling factor 1; CPM, *N*-[7-(diethylamino)-4-methylcoumarin-3-yl]maleimide; FM, fluorescein-5-maleimide; TNP-ATP, 2′(3′)-(trinitrophenyl)adenosine 5′-triphosphate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; $CF_1(-\delta,\epsilon)$, CF_1 lacking the δ- and ε-polypeptides.

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and quinine sulfate was from Aldrich Chemical Co. All other chemicals were high-quality commercial grades, and all solutions were prepared from deionized water.

Enzyme. CF_1 was prepared from fresh market spinach by known procedures (Lien & Racker, 1971; Binder et al., 1978). The enzyme was stored as a precipitate in 50% ammonium sulfate, 10 mM Tris–H_2SO_4 (pH 7.1), 1 mM EDTA, and 1 mM ATP at 4 °C. Enzyme with a fluorescence ratio, 305 nm/340 nm (excitation 280 nm), greater than 1.7 was used. The protein concentrations were determined by use of an extinction coefficient of 0.483 cm^2/mg at 277 nm (Bruist & Hammes, 1981). A molecular weight of 400 000 was assumed for CF_1 (Moroney et al., 1983). Latent CF_1 was activated by heating at 63 °C for 4 min in 10 mM dithiothreitol, 50 mM Tris-HCl (pH 8.0), 40 mM ATP, and 1 mM EDTA (Moroney et al., 1983). The ATPase activity was assayed for 5 min at 37 °C in 50 mM Tris-HCl (pH 8.0), 5 mM ATP, 5 mM $CaCl_2$, and 2 mM EDTA, with P_i being determined spectrophotometrically (Taussky & Shorr, 1953). Enzyme with an activity >15 μmol/(mg·min) was used.

$CF_1(-\delta,\epsilon)$ was prepared from CF_1 with a DEAE-cellulose column (Richter et al., 1984); 10–15 mg of CF_1 was bound to a 1 × 10 cm DEAE-cellulose column equilibrated at room temperature with 25 mM Tris-HCl (pH 7.9), 2 mM ATP, and 5 mM dithiothreitol. The column was washed with 25 mL of the same buffer. The δ- and ε-polypeptides were eluted by washing the column with 100 mL of 25 mM Tris-HCl (pH 7.5) containing 5 mM ATP, 5 mM dithiothreitol, 30% glycerol (v/v), 20% ethanol (v/v), and 0.1 M NaCl. The column was again washed with 25 mL of 25 mM Tris-HCl (pH 7.9) and 2 mM ATP. Finally, $CF_1(-\delta,\epsilon)$ was eluted with the same solution containing 0.4 M NaCl. Approximately 40–50% of the CF_1 applied to the column was recovered. Removal of δ- and ε-polypeptides was complete, as checked by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Chua, 1980). $CF_1(-\delta,\epsilon)$ was stored as a precipitate in 50% ammonium sulfate, 10 mM Tris–H_2SO_4 (pH 7.1), and 1 mM EDTA at 4 °C for a maximum of 1 week. For all experiments, this precipitate was centrifuged at 10 000 rpm in an Eppendorf centrifuge for 5 min, and the pellet was taken up in 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 1 mM EDTA. The enzyme was activated by incubation with 20–30 mM dithiothreitol for 1 h at room temperature (~21 °C).

The ATPase activity of $CF_1(-\delta,\epsilon)$ was assayed exactly as for CF_1 in 50 mM Tris-HCl (pH 8.0), 5 mM ATP, 2 mM EDTA, and 5 mM $CaCl_2$ or $MgCl_2$ for 5 min at 37 °C. $CF_1(-\delta,\epsilon)$ concentrations were determined by absorbance measurements, with the same extinction coefficient as CF_1. The absorption spectra of CF_1 and $CF_1(-\delta,\epsilon)$ were essentially identical. A molecular weight of 370 000 was calculated for $CF_1(\delta,\epsilon)$, based on molecular weights of 400 000 for CF_1, 17 500 for δ, and 13 500 for ε, and a polypeptide chain stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$ (Moroney et al., 1983).

Nucleotide Binding Sites. For nucleotide binding measurements, $CF_1(-\delta,\epsilon)$ was taken up in 50 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA, and dissociable nucleotides were removed by passage through one to two Sephadex G-50 centrifuge column(s) equilibrated with the same buffer (Penefsky, 1977).

The nondissociable ADP site of activated $CF_1(-\delta,\epsilon)$, site 1, was labeled with radioactive nucleotide by incubating 2–5 μM $CF_1(-\delta,\epsilon)$ with 200 μM [^{3}H]ATP. The stoichiometry of bound nucleotide was measured as a function of time by passing aliquots of the reaction mixture through two to three centrifuge columns. Protein concentrations were determined by absorbance measurements and nucleotide concentrations by measuring the amount of radioactivity with a Beckman LS200 scintillation counter.

The nucleotide binding site that binds MgATP very tightly on CF_1 (site 2) was labeled on $CF_1(-\delta,\epsilon)$ by incubating the enzyme with 4 mM $MgCl_2$ and 200 μM either of [^{32}P]ATP or of [^{3}H]ATP for 10 min. It was then passed through two centrifuge columns to remove unbound nucleotide.

The rate of dissociation of enzyme-bound MgATP was measured by allowing the labeled enzyme to incubate in 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 1 mM EDTA, with or without 200 μM nonradioactive ATP, at room temperature; for some experiments, 4 mM $MgCl_2$ was added to the reaction mixture. At timed intervals, an aliquot was passed through one to two centrifuge column(s) to remove dissociated nucleotide. The stoichiometry of bound nucleotide was then determined by measuring protein concentrations with absorbance and nucleotide concentrations with scintillation counting. As a control, the same experiment was performed with latent CF_1.

Steady-State Kinetics. The steady-state initial velocity of CaATP hydrolysis catalyzed by $CF_1(-\delta,\epsilon)$ was measured as a function of the substrate concentration. The assays were carried out at room temperature in 50 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 0.5 mM EDTA. The concentration of free Ca^{2+} was fixed at 5 mM; in some cases, CaADP was added to the reaction mixture. The ATP hydrolyzed was assayed by measuring the amount of [^{32}P]P_i formed. One milliliter of reaction mixture was quenched with 0.1 mL of 50% trichloroacetic acid, and the [^{32}P]P_i formed was measured by reacting the quenched reaction mixture with an equal amount of 16% ammonium molybdate solution in 10 N HCl, followed by extraction of the P_i with a 1:1 isobutyl alcohol/benzene mixture (v/v). The radioactivity in the organic layer was determined by scintillation counting (Leckband & Hammes, 1987). To ensure that initial velocities were measured, the amount of ATP hydrolyzed was less than 5% in all cases.

Chemical Modification of $CF_1(-\delta,\epsilon)$. $CF_1(-\delta,\epsilon)$, as prepared by DEAE-cellulose column chromatography, has much of the γ-disulfide reduced. Therefore, covalent labeling of $CF_1(-\delta,\epsilon)$ by maleimides results in labeling of the exposed γ-sulfhydryl ("dark" site) and variable amounts of the reduced γ-disulfide. In order to label specifically the γ-sulfhydryl (dark site), the reduced γ-disulfide first was oxidized by incubating 2–5 μM $CF_1(-\delta,\epsilon)$ with 2–5 μM $CuCl_2$ in 50 mM Tris-HCl (pH 8.0) for 1 h (Arana & Vallejos, 1982), followed by centrifugation through a G-50 Sephadex column equilibrated with 50 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA. The loss in activity of the enzyme after oxidation of the γ-disulfide could be regained fully be reduction with 30 mM dithiothreitol for 1 h at room temperature.

The exposed γ-sulfhydryl on the oxidized enzyme was covalently labeled by incubating 2–5 μM $CF_1(-\delta,\epsilon)$ with 50 μM CPM in 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 1.0 mM EDTA for 10–12 min (Snyder & Hammes, 1984, 1985). Unreacted probe was removed by passing the reaction mixture through two to three centrifuge columns equilibrated with the above buffer. Polypeptide chain specificity of labeling was checked by the fluorescence of sodium dodecyl sulfate–polyacrylamide gels illuminated with ultraviolet light.

Before the γ-disulfide site was labeled, all exposed sulfhydryls on the CPM-modified $CF_1(-\delta,\epsilon)$ were blocked by reacting the enzyme with 2 mM *N*-ethylmaleimide for 15 min in the above buffer. The γ-disulfide was then reduced by incubating the enzyme with 30 mM dithiothreitol for 1 h.

Excess dithiothreitol was removed by passage of the enzyme through two centrifuge columns equilibrated with the same buffer. The enzyme was next incubated with 50 μM FM for 10–12 min. Excess probe was removed by passage of the enzyme through two to three centrifuge columns. The polypeptide chain specificity of labeling was checked by the fluorescence of sodium dodecyl sulfate–polyacrylamide gels.

The nucleotide sites were labeled with TNP-ATP by known procedures (Cerione & Hammes, 1982; Snyder & Hammes, 1984); 2–5 μM CPM-labeled enzyme was incubated with 50–100 μM TNP-ATP in 50 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA for 1–1.5 h. Excess probe was removed through column centrifugation. Under these conditions, the tight ADP site (site 1) was labeled. Simultaneous labeling of site 1 and the MgATP site (site 2) was achieved by adding 4 mM $MgCl_2$ to the reaction mixture.

Labeling stoichiometries and concentrations of probes were calculated by using extinction coefficients of 3.02×10^4 M^{-1} cm^{-1} at 387 nm for CPM (Sippel, 1981), 7.08×10^4 M^{-1} cm^{-1} at 495 nm for FM (Cerione et al., 1983), 2.64×10^4 M^{-1} cm^{-1} at 408 nm for free TNP-ATP (Hiratsuka & Uchida, 1973), and 2.51×10^4 M^{-1} cm^{-1} at 418 nm for TNP-ATP bound to enzyme (Cerione & Hammes, 1982). In all cases, corrections were made for probe absorbance at 277 nm, probe–probe spectral overlaps, and light scattering due to enzyme.

Spectroscopic Measurements. Absorbance measurements were made with a Cary 118 spectrophotometer. Steady-state fluorescence measurements were made with a Hitachi Perkin-Elmer MPF-44B fluorescence spectrophotometer equipped with corrected spectrum and polarization accessories. Steady-state polarization measurements were corrected for unequal transmission of horizontally and vertically polarized light by the emission monochromator grating (Cerione & Hammes, 1982). Quantum yields for the fluorescent labeled enzyme samples were measured by a comparative method (Parker & Reese, 1966). The fluorescence standard used was quinine sulfate in 0.1 N H_2SO_4, which was assumed to have a quantum yield of 0.70 (Scott et al., 1970).

Measurements of fluorescence resonance energy transfer were made after correcting the donor fluorescence for protein light scattering, probe absorbance, and stoichiometry of acceptor binding. Contributions of inner filter effects to the steady-state fluorescence measurements were determined from the observed decrease in fluorescence of a dithiothreitol–CPM adduct. The efficiency of energy transfer, E, is given by

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

where Q_{DA} and Q_D are the donor quantum yields in the presence and absence of the energy acceptor, respectively. The distance R_i between a specific donor–acceptor pair is given by (Förster, 1959)

$$E = [\sum_{i=1}^{N_A}(R_0/R_i)^6]/[1 + \sum_{i=1}^{N_A}(R_0/R_i)^6] \quad (2)$$

where N_A is the number of accpetors contributing to the quenching of a single donor and R_0 is obtained from

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

Here, n is the refractive index of the medium, J is the spectral overlap integral, and κ^2 is an orientation factor for dipolar coupling between donors and acceptors. The value of κ^2 was assumed to be $^2/_3$, the dynamic average. In cases where multiple acceptors were present, all but one of the distances had been previously measured, so that eq 2 contained only one unknown.

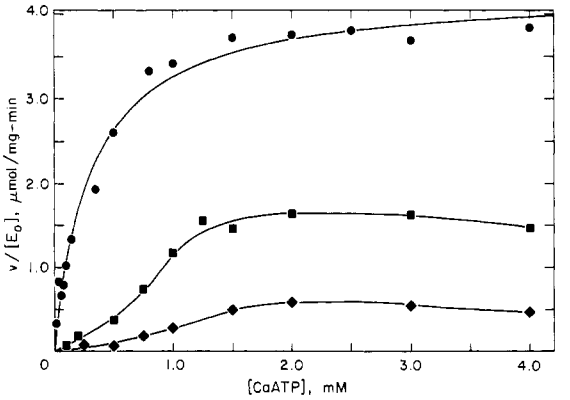

FIGURE 1: Plot of the initial steady-state velocity of CaATP hydrolysis vs CaATP concentrations. The ADP concentrations were 0 (●), 250 (■), and 500 μM (♦). The assays were carried out with 0.05–0.10 μM dithiothreitol-activated $CF_1(-\delta,\epsilon)$ in 50 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 0.5 mM EDTA at 21 °C. The lines have no theoretical significance.

RESULTS

Steady-State Kinetics. $CF_1(-\delta,\epsilon)$ is an active Ca^{2+}-ATPase. Treatment with 20–30 mM dithiothreitol for 1 h at room temperature increased the ATPase activity. Typical activities after dithiothreitol treatment were between 20 and 30 μmol of P_i/(mg·min). The Mg^{2+}-ATPase activity was, however, very low [2–3 μmol of P_i/(mg·min) in 50 mM Tris-HCl (pH 8.0), 5 mM ATP, 5 mM $MgCl_2$, and 1 mM EDTA, at 37 °C].

Plots of the steady-state initial velocities of CaATP hydrolysis vs the substrate concentrations are shown in Figure 1. ADP and high concentrations of ATP inhibited the enzyme. No attempt was made to fit these data to a specific mechanism.

Nucleotide Binding Sites. Incorporation of [^{3}H]ATP into the nondissociable ADP site of $CF_1(-\delta,\epsilon)$, site 1, was measured as a function of time. The stoichiometry of bound [^{3}H]ATP increased with the time of incubation of the enzyme with the nucleotide, leveling off at 1.2 mol of [^{3}H]ATP/mol of $CF_1(-\delta,\epsilon)$ after 1 h. The half-time was ~10 min. Incubation for longer periods (up to 24 h) did not increase the stoichiometry significantly. When the labeled enzyme was incubated with 500 μM nonradioactive ATP and 4 mM $CaCl_2$, the stoichiometry decreased to 0.2–0.3 mol of [^{3}H]ATP/mol of $CF_1(-\delta,\epsilon)$ within 4 min; after 1 h, the label was completely lost. Therefore, site 1 on $CF_1(-\delta,\epsilon)$ binds ADP tightly, and nucleotide exchange occurs in the presence of added nucleotide.

When $CF_1(-\delta,\epsilon)$ was incubated with [^{3}H]ATP or [γ-^{32}P]-ATP in the presence of Mg^{2+}, sites 1 and 2 were both labeled. The stoichiometry of bound nucleotide was 2.2–2.5 mol/mol of enzyme after 5–10 min of incubation. This indicates that the rate of exchange of ATP in site 1 is increased in the presence of Mg^{2+}; site 2 is labeled in less than 1 min, considerably faster than site 1. Site 2 on CF_1 is known to bind MgATP tightly. The rate of dissociation of bound Mg[^{3}H]-ATP from site 2 was measured by incubating the labeled enzyme in labeling buffer, in the presence of 200 μM nonradioactive ATP, in the presence of 4 mM Mg^{2+}, and in the presence of 200 μM ATP and 4 mM Mg^{2+}. The results are shown in Figure 2. In the presence of medium ATP, the radioactive nucleotide in site 1 exchanged completely, as expected. The radioactive nucleotide on site 2 slowly dissociated, the dissociation being faster in the presence of added nucleotide. When EDTA was excluded from the buffer, the loss of the label was somewhat slower, but the overall trend was similar. The rate of dissociation of MgATP from site 2 also was studied in the same buffer containing 4 mM Mg^{2+}. In

Table I: Energy Transfer Parameters for $CF_1(-\delta,\epsilon)$[a]

donor	location	fluorescence max (nm)	Q_D	P	acceptor	location	absorbance max (nm)	R_0 (Å)
CPM	dark	465	0.76	0.38	TNP-ATP	N_1–N_3	418/480	45.5
CPM	dark	465	0.76	0.38	FM	DiSH	495	51.0

[a] Abbreviations: dark, γ-sulfhydryl site; N_1–N_3, nucleotide sites 1–3; DiSH, γ-disulfide site; P, steady-state polarization measured at fluorescence excitation and emission maxima. R_0 was calculated with eq 3 and the spectral properties of donor and acceptor species.

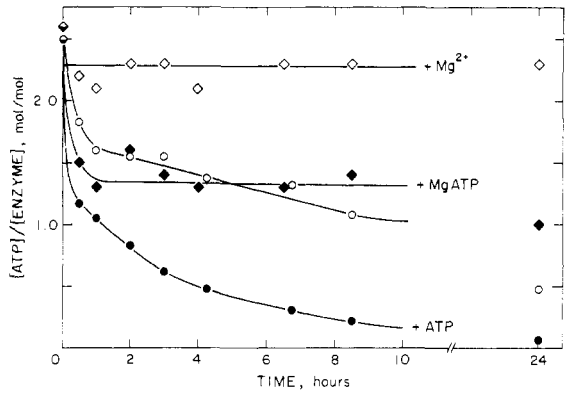

FIGURE 2: Plot of the stoichiometry of Mg[^{3}H]ATP on $CF_1(-\delta,\epsilon)$ vs time. Experiments were carried out in 50 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA at 21 °C. The labeled enzyme (1–3 μM) was incubated in this buffer (○); plus 4 mM Mg^{2+} (◇); plus 200 μM nonradioactive ATP (●); plus 4 mM Mg^{2+} and 200 μM nonradioactive ATP (◆). The lines have no theoretical significance.

this case, in the absence of nonradioactive ATP, no significant loss in the enzyme-bound MgATP occurred, whereas in the presence of 4 mM Mg^{2+} and 0.2 mM ATP, the stoichiometry decreased to 1.0 mol of nucleotide/mol of $CF_1(-\delta,\epsilon)$ within 1 h (Figure 2); no further loss occurred over 24 h. No loss in enzyme activity was observed during these measurements. Therefore, in the presence of Mg^{2+}, site 2 on $CF_1(-\delta,\epsilon)$ retains MgATP, even in the presence of added nucleotide. Essentially identical results were obtained with CF_1.

Structural Mapping. Fluorescence resonance energy transfer has been used to map distances between different sites on CF_1 [cf. Snyder and Hammes (1984, 1985)]. In the present study, intramolecular distances in $CF_1(-\delta,\epsilon)$ were determined to check whether removal of the two smaller subunits, δ and ε, leads to major structural changes in the $\alpha_3\beta_3\gamma$ enzyme.

The exposed sulfhydryl (dark site) on the γ-polypeptide of the oxidized $CF_1(-\delta,\epsilon)$ was covalently modified with CPM. Before covalent modification of the enzyme, the reduced γ-disulfide was oxidized with $CuCl_2$. The effectiveness of the oxidation was checked by labeling $CF_1(-\delta,\epsilon)$, before and after oxidation, with CPM or FM; before oxidation, 2–2.5 mol of the maleimide derivative was bound per mole of $CF_1(-\delta,\epsilon)$, whereas after oxidation the stoichiometry was about 1.

The fluorescence properties of CPM at the exposed sulfhydryl of the γ-polypeptide on $CF_1(-\delta,\epsilon)$, summarized in Table I, are very similar to those of CPM at the same site on CF_1 (Snyder & Hammes, 1985). The high polarization indicates that even after removal of the δ and ε subunits, the label is in a very restricted environment. Labeling stoichiometries were typically between 0.5 and 0.7 mol of CPM/mol of $CF_1(-\delta,\epsilon)$; a higher stoichiometry often resulted in nonspecific labeling of the α- and β-polypeptides.

The fluorescence emission spectrum of CPM on the γ-polypeptide sulfhydryl overlaps the absorption spectrum of both TNP-ATP at the nucleotide sites and FM at the γ-disulfide (Synder & Hammes, 1985). The γ-disulfide was labeled with FM; stoichiometries ranged from 1.0 to 1.5 mol of FM/mol of $CF_1(-\delta,\epsilon)$. No correlation was found between CPM stoichiometries at the γ-sulfhydryl and FM stoichiometries at the γ-disulfide. The steady-state polarization of FM at the γ-disulfide was 0.17, somewhat less than the value obtained for CF_1, 0.21. The efficiency of energy transfer between the CPM-labeled sulfhydryl and the FM-labeled γ-disulfide was measured by comparing the fluorescence of the $CF_1(-\delta,\epsilon)$ containing FM to that containing no FM; the quenching ratio was normalized with respect to the protein concentrations. The observed efficiency was then transformed into the value expected for one acceptor per enzyme molecule by use of eq 2 and the measured acceptor stoichiometry. The transformation was done for two limiting cases. In the first, the labeling of the γ-disulfide was assumed to be random, such that the fraction of enzyme molecules containing one, two, or no FM molecule(s) was determined by the binomial distribution. In the second case, simultaneous labeling of both reduced sulfhydryls was assumed to be severly restricted such that for FM stoichiometries <1 mol/mol, all $CF_1(-\delta,\epsilon)$ contained only one FM label, and for stoichiometries >1 mol/mol, at least one FM label. the transformed efficiencies for random and restricted labeling were 0.80 and 0.64/mol of FM, respectively, which yielded a separation of 40–46 Å between the γ-sulfhydryl and disulfide sites.

The nucleotide sites on the CPM-modified $CF_1(-\delta,\epsilon)$ were labeled with TNP-ATP. Only nucleotide site 1 was labeled when the enzyme was incubated with TNP-ATP in the absence of Mg^{2+}. Stoichiometries were 1.0–1.3 mol of TNP-ATP/mol of $CF_1(-\delta,\epsilon)$, somewhat higher than for control CF_1. The energy transfer efficiency between the γ-sulfhydryl and nucleotide site 1 was measured by comparing the fluorescence of the CPM-labeled $CF_1(-\delta,\epsilon)$ containing TNP-ATP to that containing no TNP-ATP; the quenching ratio was normalized with respect to the protein concentrations. A transfer efficiency of 0.42/mol of bound TNP-ATP was obtained. This corresponds to a distance of 48 Å between the γ-sulfhydryl and nucleotide site 1.

Nucleotide sites 1 and 2 were simultaneously labeled with TNP-ATP in the presence of Mg^{2+} in the buffer. Stoichiometries ranged between 2.0 and 2.3 mol of TNP-ATP/mol of $CF_1(-\delta,\epsilon)$. There was no loss in the TNP-ATP label at site 2 over the course of 6–8 h if Mg^{2+} was kept in the buffer. The combined energy transfer efficiency between the γ-sulfhydryl and nucleotide sites 1 and 2 was then measured as before. A value of 0.65 was obtained. By use of eq 2, the distance calculated above for site 1, and the combined energy transfer efficiency with both sites 1 and 2 occupied, a distance of 45 Å between nucleotide site 2 and the γ-sulfhydryl was obtained.

The efficiency of energy transfer between the γ-sulfhydryl and nucleotide site 3 was obtained by titrating the CPM-labeled enzyme with TNP-ATP and measuring the decrease in CPM fluorescence. The enzyme was saturated with TNP-ATP at nucleotide sites 1 and 2 before the titration was performed, thus ensuring that the observed quenching was due solely to binding at site 3. Figure 3 shows the titration as a plot of the quenching ratio vs the total concentration of TNP-ATP. The data were fit to the equation:

$$Q_{DA}/Q_D = 1 - E[EL]/[E_0] \quad (4)$$

where E is the transfer efficiency when site 3 is fully occupied,

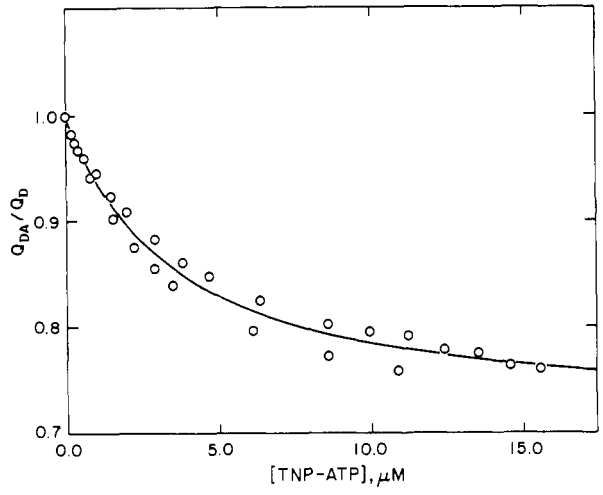

FIGURE 3: Plot of the fluorescence quenching of CPM labeled at the γ-sulfhydryl (dark site) of $CF_1(-\delta,\epsilon)$ vs the total TNP-ATP concentration. Q_{DA} and Q_D are the quantum yields in the presence and absence of TNP-ATP bound to site 3, respectively. Sites 1 and 2 were saturated with TNP-ATP prior to the titration of site 3. Titrations were carried out with ~0.5 μM $CF_1(-\delta,\epsilon)$ in 50 mM NaCl, 10 mM Tris-HCl (pH 8.0), 4 mM $MgCl_2$, and 1 mM EDTA at 21 °C. The curve is a nonlinear least-squares fit of the data to eq 4 as described in the text.

[EL] is the concentration of bound ligand, and $[E_0]$ is the total enzyme concentration. The concentration of EL can be calculated from $[E_0]$, the dissociation constant K_d, and the total ligand concentration $[L_0]$ by using

$$2[EL]/[E_0] = (1 + [L_0]/[E_0] + K_d/[E_0]) - \{(1 + [L_0]/[E_0] + K_d/[E_0])^2 - 4[L_0]/[E_0]\}^{1/2} \quad (5)$$

A nonlinear least-squares fit of the data gave $K_d = 3.2 \pm 0.2$ μM and $E = 0.29 \pm 0.02$. The total energy transfer efficiency for all three nucleotide sites containing 1 mol of bound TNP-ATP is 0.75. If this efficiency is combined with the distances to sites 1 and 2 already calculated and eq 2, a distance of 45 Å between nucleotide site 3 and the γ-sulfhydryl can be calculated.

Discussion

The kinetic data in Figure 1 indicate that $CF_1(-\delta,\epsilon)$ resembles CF_1 closely in the substrate dependence of the steady-state rate of ATP hydrolysis (Cantley & Hammes, 1975); i.e., removal of the two smaller polypeptides has no effect on the steady-state kinetics. In contrast to CF_1, $CF_1(-\delta,\epsilon)$ is an active ATPase; this is partially caused by the removal of the regulatory polypeptide, ε, and partially by the reduction of the γ-disulfide during the enzyme preparation. It has been suggested that removal of ε from CF_1 makes the γ-disulfide particularly susceptible to reduction (Richter et al., 1985).

Nucleotide binding studies reveal that the nucleotide sites on $CF_1(-\delta,\epsilon)$ have essentially the same characteristics as those on CF_1. Stoichiometries of bound nucleotids on $CF_1(-\delta,\epsilon)$ were consistently higher than those on CF_1 (e.g., 1.2 vs 1.1 mol/mol and 2.2–2.5 vs 2.1 mol/mol); this may have been caused by an overestimation of the extinction coefficient of $CF_1(-\delta,\epsilon)$. Alternatively, there may be more sites on $CF_1(-\delta,\epsilon)$ that bind nucleotides tightly. The tightness of binding of MgATP at nucleotide site 2 was studied. It can be concluded from Figure 2 that bound MgATP slowly dissociates from the enzyme over a period of hours if Mg^{2+} is excluded from the buffer. This dissociation occurs both in the presence and in the absence of nonradioactive ATP. EDTA facilitates this dissociation but is not the sole cause of it since even in the absence of EDTA and Mg^{2+}, some of the bound MgATP dissociated. The binding of MgATP at site 2 is, however, tight if Mg^{2+} is present in the buffer. Both CF_1 and $CF_1(-\delta,\epsilon)$ were found to be similar in this respect.

Chemical modification of $CF_1(-\delta,\epsilon)$ was performed with fluorescent maleimides exactly as for CF_1 (Snyder & Hammes, 1985). The steady-state polarizations of CPM at the γ-sulfhydryl (dark site) and of FM at the γ-disulfide on $CF_1(-\delta,\epsilon)$, when compared to the corresponding values on CF_1, indicate that after removal of the δ- and ε-polypeptides, the label at the γ-disulfide is in a slightly less restrictive environment, whereas there is no change in the environment of the label at the sulfhydryl site. In contrast to CF_1 (Snyder & Hammes, 1985), no correlation was found between the stoichiometries of bound maleimides at the γ-sulfhydryl and γ-disulfide sites. This again may reflect a less hindered environment around the γ-disulfide as a result of removing the ε-polypeptide.

Fluorescence resonance energy transfer measurements yield a separation of 40–46 Å between the γ-sulfhydryl (dark site) and the γ-disulfide on $CF_1(-\delta,\epsilon)$. The distance between the same sites on CF_1 is 41–46 Å (Snyder & Hammes, 1985). This indicates that there is no structural change in the γ-polypeptide as a result of removal of the δ and ε subunits. The distances between the γ dark site and nucleotide sites 1, 2, and 3 on $CF_1(-\delta,\epsilon)$ are 48, 45, and 45 Å, respectively. On CF_1, the corresponding distances are 48, 43, and 47 Å (Snyder & Hammes, 1985). The measured distances on CF_1 and $CF_1(-\delta,\epsilon)$ are within 5% of each other. As previously discussed (Richter et al., 1985), the experimental uncertainty in the distances is about ±10%. The dissociation constant of nucleotide binding at site 3 on $CF_1(-\delta,\epsilon)$, as obtained from the TNP-ATP titration (Figure 3), is in the range of values found for CF_1 (Cantley & Hammes, 1975; Bruist & Hammes, 1981; Cerione & Hammes, 1982; Snyder & Hammes, 1984, 1985). Comparison of the distances measured on $CF_1(-\delta,\epsilon)$ to those on CF_1 indicates no major structural change in the $\alpha_3\beta_3\gamma$ enzyme occurs when δ and ε are removed.

The results obtained in this study indicate that as far as the ATPase activity and structure are concerned, $CF_1(-\delta,\epsilon)$ is very similar to CF_1. The ε subunit clearly plays an inhibitory role since its removal activates the enzyme. The δ-polypeptide does not play any apparent role in the ATPase activity of CF_1. $CF_1(-\delta,\epsilon)$ is a structurally simpler enzyme for study than CF_1. Also, in contrast to the highly asymmetric shape of CF_1, caused by the δ-polypeptide, $CF_1(-\delta,\epsilon)$ is approximately spherical (Schinkel & Hammes, 1986). It might, therefore, be possible to obtain crystals of $CF_1(-\delta,\epsilon)$ suitable for X-ray crystallography.

Registry No. ATPase, 9000-83-3; CPM, 93111-28-5; FM, 75350-46-8; TNP-ATP, 61368-63-6; ADP, 58-64-0; MgATP, 1476-84-2; ATP, 56-65-5; CaATP, 15866-84-9.

References

Admon, A., & Hammes, G. G. (1987) *Biochemistry 26*, 3193–3197.

Arana, J. L., & Vallejos, R. H. (1982) *J. Biol. Chem. 257*, 1125–1127.

Bar-Zvi, D., & Shavit, N. (1982) *Biochim. Biophys. Acta 681*, 451–458.

Binder, A., Jagendorf, A., & Ngo, E. (1978) *J. Biol. Chem. 253*, 3094–3100.

Bruist, M. F., & Hammes, G. G. (1981) *Biochemistry 20*, 6298–6305.

Bruist, M. F., & Hammes, G. G. (1982) *Biochemistry 21*, 3370–3377.

Cantley, L. C., & Hammes, G. G. (1975) *Biochemistry 14*, 2968-2975.
Cerione, R. A., & Hammes, G. G. (1982) *Biochemistry 21*, 745-752.
Cerione, R. A., McCarty, R. E., & Hammes, G. G. (1983) *Biochemistry 22*, 769-776.
Chua, N. H. (1980) *Methods Enzymol. 69*, 434-446.
Czarnecki, J. J., Abbott, M. S., & Selman, B. R. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 7744-7748.
Förster, Th. (1959) *Discuss. Faraday Soc. 27*, 7-17.
Hiratsuka, T., & Uchida, K. (1973) *Biochim. Biophys. Acta 320*, 635-647.
Kambouris, N. K., & Hammes, G. G. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1950-1953.
Leckband, D., & Hammes, G. G. (1987) *Biochemistry 26*, 2306-2312.
Lien, S., & Racker, Ed. (1971) *Methods Enzymol. 23*, 547-555.
McCarty, R. E., & Moroney, J. V. (1985) *Enzymes Biol. Membr. 4*, 383-413.
McCarty, R. E., & Hammes, G. G. (1987) *Trends Biochem. Sci. (Pers. Ed.) 12*, 234-237.
Moroney, J. V., Lopresti, L., McEwen, B. F., McCarthy, R. E., & Hammes, G. G. (1983) *FEBS Lett. 158*, 58-62.
Nalin, C. M., Snyder, B., & McCarty, R. E. (1985) *Biochemistry 24*, 2318-2324.
Parker, C. A., & Reese, W. T. (1966) *Analyst (London) 85*, 587-600.
Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891-2899.
Richter, M. L., Patrie, W. J., & McCarty, R. E. (1984) *J. Biol. Chem. 259*, 7371-7373.
Richter, M. L., Snyder, B., McCarty, R. E., & Hammes, G. G. (1985) *Biochemistry 24*, 5755-5763.
Schinkel, J. E., & Hammes, G. G. (1986) *Biochemistry 25*, 4066-4071.
Scott, T. G., Spencer, R. D., Leonard, N. J., & Weber, G. (1970) *J. Am. Chem. Soc. 92*, 687-695.
Sippel, T. O. (1981) *J. Histochem. Cytochem. 29*, 1377-1381.
Snyder, B., & Hammes, G. G. (1984) *Biochemistry 23*, 5787-5795.
Snyder, B., & Hammes, G. G. (1985) *Biochemistry 24*, 2324-2331.
Taussky, H. H., & Shorr, E. (1953) *J. Biol. Chem. 202*, 675-685.

Conformational States of *N*-Acylalanine Dithio Esters: Correlation of Resonance Raman Spectra with Structures†

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ABSTRACT: The conformational states of *N*-acylalanine dithio esters, involving rotational isomers about the RC(=O)NH—CH(CH_3) and NHCH(CH_3)—C(=S) bonds, are defined and compared to those of *N*-acylglycine dithio esters. The structure of *N*-(*p*-nitrobenzoyl)-DL-alanine ethyl dithio ester has been determined by X-ray crystallographic analysis; it is a B-type conformer with the amide N atom cis to the thiol sulfur. Raman and resonance Raman (RR) measurements on this compound and for the B conformers of solid *N*-benzoyl-DL-alanine ethyl dithio ester and *N*-(β-phenylpropionyl)-DL-alanine ethyl dithio ester and its NHCH(CD_3)C(=S) and NHCH(CH_3)^{13}C(=S) analogues are used to set up a library of RR data for alanine-based dithio esters in a B-conformer state. (Methyloxycarbonyl)-L-phenylalanyl-L-alanine ethyl dithio ester crystallizes in an A-like conformational state wherein the alanine N atom is nearly cis to the thiono S atom (C=S) [Varughese, K. I., Angus, R. H., Carey, P. R., Lee, H., & Storer, A. C. (1986) *Can. J. Chem. 64*, 1668-1673]. RR data for this solid material in its isotopically unsubstituted and CH(CD_3)C(=S) and CH(CH_3)^{13}C(=S) forms provide information on the RR signatures of alanine dithio esters in A-like conformations. RR spectra are compared for the solid compounds, for *N*-(*p*-nitrobenzoyl)-DL-alanine, *N*-(β-phenylpropionyl)-DL-alanine, and (methyloxycarbonyl)-L-phenylalanyl-DL-alanine ethyl dithio esters, and for several ^{13}C=S- and CD_3-substituted analogues in CCl_4 or aqueous solutions. The RR data demonstrate that the alanine-based dithio esters take up A, B, and C_5 conformations in solution. The RR spectra of these conformers are clearly distinguishable from those for the same conformers of *N*-acylglycine dithio esters. However, the crystallographic and spectroscopic results show that the conformational properties of *N*-acylglycine and *N*-acylalanine dithio esters are very similar.

Resonance Raman (RR) data for *N*-acylglycine dithioacyl papains have provided detailed information on the conformation of the covalently bound substrates (Carey & Storer, 1984, 1985). Spectral interpretation for these enzyme–substrate intermediates has been greatly aided by the study of model compounds in the form of *N*-acylglycine ethyl dithio esters. Many of the model compounds could be crystallized as single crystals suitable for X-ray crystallographic analysis, and since the crystals could also be examined by Raman and RR spectroscopy, it was possible to construct a library of precise structure–spectra correlations for use in interpreting the dithioacyl papain RR spectra.

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