Reid, B. R., Banks, K., Flynn, P., & Nerdal, W. (1989) *Biochemistry 28*, 10001-10007.
Rinkel, L. J., van der Marel, G. A., van Boom, J. H., & Altona, C. (1987) *Eur. J. Biochem. 163*, 275-286.
Sarma, M. H., Dhingra, M. M., Gupta, G., & Sarma, R. H. (1985) *Biochem. Biophys. Res. Commun. 131*, 269-276.
Sarma, R. H., Sarma, M. H., & Gupta, G. (1986) *Proceedings of the 4th Conversation in the Discipline of Biomolecular Stereodynamics* (Sarma, R. H., & Sarma, M. H., Eds.) pp 157-177, Adenine, New York.
Sarma, M. H., Gupta, G., & Sarma, R. H. (1988) *Biochemistry 27*, 3423-3432.
Sarma, M. H., Gupta, G., Garcia, A. E., Umemoto, K., & Sarma, R. H. (1990) *Biochemistry 29*, 4723-4734.
Schmitz, U., Pearlman, D. A., & James, T. L. (1991) *J. Mol. Biol. 221*, 271-292.
Srinivasan, A. R., Torres, R., Clark, W., & Olson, W. K. (1987) *J. Biomol. Struct. Dyn. 5*, 459-496.
States, D. J., Haberkorn, R. A., & Ruben, D. J. (1982) *J. Magn. Reson. 48*, 286-292.
Steitz, T. A. (1990) *Q. Rev. Biophys. 23*, 205-280.
Strauss, F., Gaillard, C., & Prunell, A. (1981) *Eur. J. Biochem. 118*, 215-222.
Trifonov, E. N. (1985) *CRC Crit. Rev. Biochem. 19*, 89-106.
Ulyanov, N. B., & Zhurkin, V. B. (1982) *Molek. Biol. (Engl. Trans.) 16*, 857-867.
Ulyanov, N. B., & Zhurkin, V. B. (1984) *J. Biomol. Struct. Dyn. 2*, 361-385.
Ulyanov, N. B., Gorin, A. A., & Zhurkin, V. B. (1989) *Proceedings of the International Conference on Supercomputing '89: Supercomputer Applications* (Kartashev, L. P., & Kartashev, S. I., Eds.) pp 368-370, International Supercomputing Institute, Inc., St. Petersburg, FL.
Umemoto, K., Sarma, M. H., Gupta, G., & Sarma, R. H. (1990a) *Biochemistry 29*, 4714-4722.
Umemoto, K., Sarma, M. H., Gupta, G., Luo, J., & Sarma, R. H. (1990b) *J. Am. Chem. Soc. 112*, 4539-4545.
Wang, J. C. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 200-203.
Wartell, R. M., & Harrel, J. T. (1986) *Biochemistry 25*, 2664-2671.
Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, Wiley, New York.
Yanagi, K., Prive, G. G., & Dickerson, R. E. (1991) *J. Mol. Biol. 217*, 201-214.
Zhou, N., Bianucci, A. M., Pattabiraman, N., & James, T. L. (1987) *Biochemistry 26*, 7905-7913.
Zhurkin, V. B., Lysov, Yu. P., & Ivanov, V. I. (1978) *Biopolymers 17*, 377-412.
Zhurkin, V. B., Poltev, V. I., & Florentiev, V. L. (1981) *Molek. Biol. (Engl. Trans.) 14*, 1116-1130.
Zhurkin, V. B., Lysov, Yu. P., Florentiev, V. L., & Ivanov, V. I. (1982) *Nucleic Acids Res. 10*, 1811-1830.
Zhurkin, V. B., Ulyanov, N. B., & Ivanov, V. I. (1988) in *Structure & Expression* (Olson, W. K., Sarma, M. H., Sarma, R. H., & Sundaralingam, M., Eds.) Vol. 3, pp 169-190, Adenine, New York.
Zhurkin, V. B., Gorin, A. A., Charakhchyan, A. A., & Ulyanov, N. B. (1990) in *Theoretical Biochemistry & Molecular Biophysics* (Beveridge, D. L., & Lavery, R., Eds.) pp 411-431, Adenine, New York.
Zhurkin, V. B., Ulyanov, N. B., Gorin, A. A., & Jernigan, R. L. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 7046-7050.

Structural Mapping of Cysteine-63 of the Chloroplast ATP Synthase β Subunit†

Kim K. Colvert,‡ Denise A. Mills, and Mark L. Richter*
Department of Biochemistry, The University of Kansas, Lawrence, Kansas 66045
Received January 17, 1991; Revised Manuscript Received January 16, 1992

ABSTRACT: The single sulfhydryl residue (cysteine-63) of the β subunit of the chloroplast ATP synthase F_1 (CF_1) was accessible to labeling reagents only after removal of the β subunit from the enzyme complex. This suggests that cysteine-63 may be located at an interface between the β and the α subunits of CF_1, although alternative explanations such as a conformational change in β brought about by its release from CF_1 cannot be ruled out. Cysteine-63 was specifically labeled with [(diethylamino)methylcoumarinyl]-maleimide, and the distance between this site and trinitrophenyl-ADP at the nucleotide binding site on β was mapped using fluorescence resonance energy transfer. Cysteine-63 is located in a hydrophobic pocket, 42 Å away from the nucleotide binding site on β.

The chloroplast coupling factor 1 (CF_1)[1] utilizes the energy of a transmembrane proton gradient to catalyze ATP synthesis. CF_1 is comprised of five different subunits designated α-ϵ in order of decreasing molecular weight. The probable subunit stoichiometry is 3α, 3β, 1γ, 1δ, and 1ϵ (McCarty & Moroney, 1985). The two larger subunits, α and β, are involved in nucleotide binding and catalysis. Attachment of photoaffinity nucleotide analogues located at known nucleotide binding/catalytic sites on CF_1 results primarily in labeling of the β

† This work was supported by grants from the National Science Foundation (DMB-8805048) and from The University of Kansas (GRF 3009).
* To whom correspondence should be addressed.
‡ Recipient of an NSF research opportunity award. Permanent address: Department of Physical Sciences, Ferris State University, Big Rapids, MI 49307.

[1] Abbreviations: CF_1, chloroplast coupling factor 1; TNP-ATP and TNP-ADP, 2′,3′-*O*-(2,4,6-trinitrophenyl) derivatives of ATP and ADP; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Tris, tris(hydroxymethyl)aminomethane; CPM, *N*-[4-[7-(diethylamino)-4-methylcoumarin-3-yl]]maleimide.

0006-2960/92/0431-3930$03.00/0

subunits (Admon & Hammes, 1987), suggesting that this subunit contains the active site. However, under certain conditions, the α subunit is also labeled by photoaffinity nucleotide analogues, suggesting that perhaps the active site resides at an interfacial region between the α and β subunits [see Nalin and Nelson (1987) and Penefsky and Cross (1990) for recent reviews].

As many as six nucleotide binding sites with markedly different properties have been identified on CF_1 (Bruist & Hammes, 1981; Cerione & Hammes, 1982; Snyder & Hammes, 1984; Leckband & Hammes, 1987; Zue et al., 1987; Shapiro & McCarty, 1988). The apparent asymmetry among the nucleotide binding/catalytic sites forms the basis for the *alternating sites* hypothesis (Boyer & Kohlbrenner, 1981) which attempts to explain the well-known cooperativity among the catalytic sites of the ATP synthases. The more recent version of this hypothesis (Boyer, 1987) suggests that three catalytic sites on the enzyme sequentially alternate between three different conformations during the catalytic cycle. Each conformation affects a change in the affinity of nucleotides for that site. Shapiro and McCarty (1988) provided some direct evidence for this model when they showed that at least two of the three nucleotide binding sites on CF_1 switch between *tight* and *loose* nucleotide binding during catalytic turnover. It has been proposed (Duncan et al., 1986) that the transfer of information between the different nucleotide binding sites on the F_1 enzymes is propagated via conformational interactions between the different α and β subunits.

We recently isolated the β subunit of CF_1 in a form which is capable of reconstituting ATPase activity to bacterial chromatophores inactivated by removal of the nascent β subunit (Richter et al., 1986). Isolation of the β subunit led to the identification of its single dissociable nucleotide binding site (Mills & Richter, 1991) which has properties essentially identical to those of catalytic site 3 (Snyder & Hammes, 1985) of CF_1.

Studies are described in this paper which show that the single sulfhydryl residue of the β subunit, located at position 63 in the amino acid sequence (Walker et al., 1984), is normally buried within the CF_1 complex but becomes accessible to labeling reagents in the medium upon isolation of β from other CF_1 subunits. Cysteine-63 of isolated β was labeled with fluorescent maleimide derivatives, and its distance to the fluorescent nucleotide analogue trinitrophenyl-ATP (TNP-ATP), located at the nucleotide binding site on β, was measured using fluorescence resonance energy transfer. The measurement indicated that the two sites are a substantial distance apart in the folded conformation of the β subunit.

Materials and Methods

Chemicals. TNP-nucleotide analogues and fluorescent maleimide derivatives were purchased from Molecular Probes Inc. Quinine sulfate was obtained from Aldrich Chemical Co., and *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-trypsin and all other nucleotides were from Sigma Chemical Co. Other chemicals were of high-quality commercial grades, and all aqueous solutions were prepared with deionized, distilled water.

Preparation of CF_1 and the β Subunit. CF_1 was prepared from fresh market spinach (Binder et al., 1978; Lien & Racker, 1971; Richter & McCarty, 1987) and stored as an ammonium sulfate precipitate. Prior to use, CF_1 was desalted on a 1.5 × 15 cm column of Sephadex G-50 or through column centrifugation (Penefsky, 1977). The β-polypeptide was prepared from CF_1 lacking the δ- and ε-polypeptides as described elsewhere (Richter et al., 1985). The protein was concentrated to >1 mg/mL over an Amicon P-30 membrane and stored at −70 °C in the presence of 20% (v/v) glycerol and 1 mM ATP.

Chemical Modification of the β Subunit. The single β-sulfhydryl was routinely modified with fluorescent maleimides by incubation of β with a 2-fold excess of the maleimide at room temperature for 15–30 min. The modification medium contained 25 mM Tricine–NaOH (pH 8.0), 1 mM ATP, and 10–20 μM β subunit. Unreacted probe was removed by gel filtration on a 1.5 × 20 cm column of Sephadex G-50. The extent of labeling by CPM was determined using the extinction coefficient of 3.02×10^4 $M^{-1}\cdot cm^{-1}$ at 387 nm (Sippel, 1981) after correction for CF_1 light scattering.

Spectroscopic Measurements. Absorbance measurements were made with a Beckman DU-70 spectrophotometer. Steady-state fluorescence measurements were made with a Perkin Elmer MPF-44B fluorescence spectrophotometer equipped with corrected spectrum and polarization accessories. The measured steady-state polarizations were corrected for unequal transmission of horizontally and vertically polarized light by the emission monochromator grating. Quantum yields for the fluorescent-labeled β samples were calculated by the comparative method of Parker and Reese (1966) using quinine sulfate as standard. The quantum yield of quinine sulfate was assumed to be 0.70 (Scott et al., 1970).

The amount of TNP-ADP bound to the β subunit at different TNP-ADP concentrations was determined from the extent of fluorescence enhancement upon TNP-ADP binding (Mills & Richter, 1991).

Fluorescence Resonance Energy Transfer Measurements. Fluorescence resonance energy transfer measurements were performed as described elsewhere (Snyder & Hammes, 1984, 1985). The donor fluorescence was corrected for the measured probe absorbance, protein light scattering, and acceptor stoichiometries. The efficiency of energy transfer was obtained from the relationship

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

where Q_{DA}/Q_D and τ_{DA}/τ_D are the ratios of the donor quantum yields and fluorescent lifetimes, respectively, in the presence (DA) and absence (D) of acceptor compounds. The distance between the donor and acceptor sites was calculated from the relationship

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

where N_A is the number of acceptors contributing to the quenching of a single donor, R_i is the distance between the donor and the ith acceptor, and R_0 is the distance at which the transfer efficiency is 0.5 for a single donor–acceptor pair. Equation 2 assumes that each is present at a stoichiometry of 1 mol of acceptor per mole of β-polypeptide. In eq 2, R_0 is given by (Forster, 1959)

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

where η is the refractive index of the medium, J is the spectral overlap integral, and κ^2 is an orientation factor for dipolar coupling between donor and acceptor. The value of κ^2 is assumed to be $^2/_3$, the dynamic average. The maximum uncertainties in the calculated distance due to this assumption are likely to be no worse than ±10% (Snyder & Hammes, 1985) but may in fact be substantially better than 10% (Richter et al., 1985). The contribution of inner filter effects and trivial transfer to the steady-state quenching ratios was measured directly by monitoring fluorescence quenching of

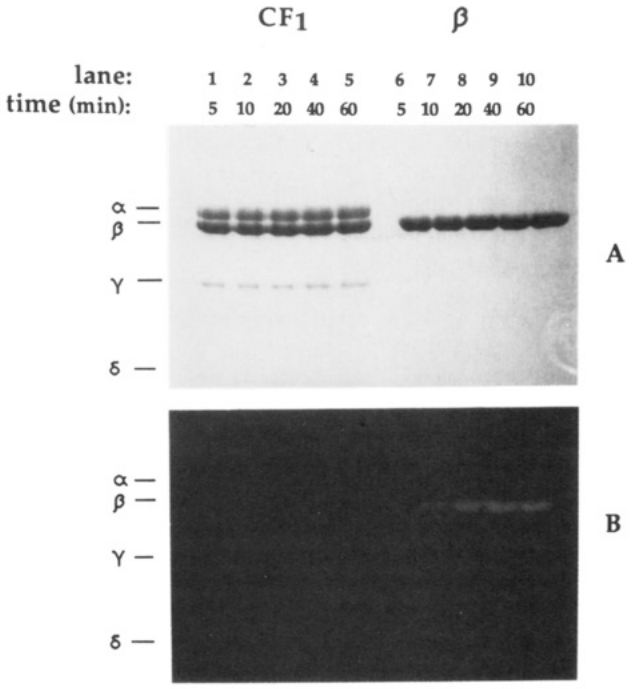

FIGURE 1: Comparison of labeling CF_1 and isolated β subunit with fluoresceinylmaleimide. To CF_1 or isolated β in 20 mM Tricine–NaOH (pH 8.0) was added a 2-fold molar excess of fluoresceinylmaleimide. Mixtures were incubated at 23 °C for the times indicated. The protein was precipitated with trichloroacetic acid and subjected to polyacrylamide (12%) gel electrophoresis in the presence of sodium dodecyl sulfate. (A) Coomassie blue staining of the gel. Each lane contained protein equivalent to 10 μg of β subunit; (B) fluorescein fluorescence monitored by placing the gel shown in (A), prior to staining, on a long-wavelength ultraviolet transilluminator.

dithiothreitol-coumarinylmaleimide due to increasing concentrations of TNP-ATP. As additional checks, the coumarinylmaleimide-labeled β subunit was treated with TNP-ADP after complete inactivation of nucleotide binding either by incubation at 50 °C for 15 min[2] or by tryptic digestion. The corrections for inner filter effects plus trivial transfer obtained by all three methods were essentially identical. For example, at a TNP-ADP concentration of 5 μM, the correction amounted to 27% of the observed steady-state fluorescence ratio.

Protein concentrations were determined by the method of Bradford et al. (1976).

Results

Sulfhydryl Modification of β Before and After Isolation. CF_1 and isolated β subunit were treated under identical conditions with fluoresceinylmaleimide and the polypeptides separated by SDS–polyacrylamide gel electrophoresis (Figure 1). Label was strongly incorporated into isolated β whereas we could not detect any label in the β subunits of whole CF_1. Thus, the β-sulfhydryl is buried within the enzyme complex, inaccessible to modifying reagents in the medium. The labeling experiment was repeated after removal of two of the three smaller subunits, δ and ϵ, from CF_1 (Richter et al., 1985). In this case, a small amount of label was associated with the β subunit (up to 0.1 mol of fluoresceinylmaleimide per mole of β), but it was 10% or less of that incorporated into isolated β under the same conditions. The labeling stoichiometry did not increase further with increased incubation time or with increased probe concentration (not shown), suggesting that

[2] A manuscript describing this work has been submitted for publication.

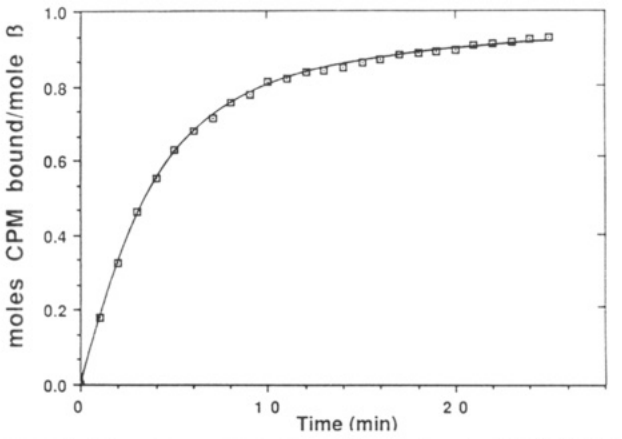

FIGURE 2: Time course of labeling of the β subunit with CPM. To the β subunit (1 mg/mL) in a solution containing 20 mM Tricine–NaOH (pH 8.0) and 1 mM ATP was added CPM to a final concentration of 40 μM. Fluorescence at 465 nm (excitation 387 nm) was monitored continuously. At the end of the titration, excess probe was removed by gel filtration (Sephadex G-50), and the amount of CPM label associated with the β subunit was determined as described under Materials and Methods.

it resulted from the presence of a small amount of free β subunit contaminating our enzyme preparations.

Time Course and Specificity of β Labeling by CPM. The spectral properties of coumarinylmaleimide (CPM) make it a suitable fluorescence donor molecule for use with TNP-nucleotide analogues as acceptors when measuring energy transfer over substantial distances (Snyder & Hammes, 1984, 1985). The extent of modification of the β subunit by CPM was measured by following the large increase in CPM fluorescence which occurs upon derivatization (Parvari et al., 1983). Labeling of β was rapid under the conditions employed, being complete within 15–20 min at pH 8 and room temperature with a small excess of probe (Figure 2). We tested a number of other fluorescent maleimides, all rather bulky nonpolar molecules, and all labeled β to a similar extent. However, labeling by fluorescent maleimides could be blocked by preincubation with micromolar amounts of the smaller, water-soluble *N*-ethylmaleimide, indicating that accessibility was not restricted to probes of low water solubility. In contrast, the amine-directed probe fluoresceinyl isothiocyanate, which readily labels a lysine residue on β (unpublished experiments), did not block labeling by CPM. These data strongly suggested that labeling by CPM was restricted to a single unique site on the β subunit, presumably the only sulfhydryl located at position 63 in the amino acid sequence (Walker et al., 1985).

The labeling specificity of CPM was further examined by chromatography of proteolytic digests of CPM-labeled β subunit. It was found that in the early stages of digestion with proteolytic enzymes (trypsin or endoprotease Glu-C), proteolytic fragments containing the CPM label aggregated, limiting further digestion. This problem was significantly reduced by including 30% (v/v) methanol or dimethyl sulfoxide in the digestion mixture.

In the experiment described in Figure 3, CPM-labeled β was exposed to endoprotease Glu-C overnight at room temperature in the presence of 30% (v/v) dimethyl sulfoxide and 50 mM sodium phosphate (pH 7.2), which allows the enzyme to cleave at both aspartate and glutamate residues (Houmard & Drapeau, 1972). The peptide containing the fluorescent probe was purified by two successive chromatography steps as described in the legend to Figure 3. The elution profile shown in Figure 3 is for the second chromatography step. One major peak was evident together with as few as one and sometimes several

Table I: Amino Acid Sequence of Fluorescent Peptide Purified from CPM-Labeled β Subunit

cycle	PTH-amino acid	pmol	position in CF_1 β sequence (Walker et al., 1985)
1	Val	39.9	33
2	Ala	34.2	34
3	Phe	32.7	35
4	Pro	36.7	36
5	Pro	35.6	37
6	Gly	34.2	38
7	Lys	6.2	39
8	Met	18.2	40
9	Pro	23.7	41
10	Asn	19.2	42
11	Ile	23.9	43
12	Tyr	9.9	44
13	Asn	14.9	45
14	Ala	17.7	46
15	Leu	18.0	47
16	Ile	15.4	48
17	Val	14.3	49
18	Lys	4.5	50
19	Gly	23.1	51
20			52
21	Asp	4.3	53
22	Thr	3.5	54
23	Ala	7.3	55
24	Gly	19.5	56
25	Gln	4.0	57
26	Pro	4.5	58
27	Met	2.4	59
28	Asn	4.0	60
29	Val	5.2	61
30	Thr	2.0	62
31			63
32	(Val)	4.0	64

small peaks or shoulders. Chromatography of dithiothreitol-reacted CPM under the same conditions also resulted in the appearance of minor peaks, indicating the presence of probe degradation products. The fluorescent material from the area indicated in Figure 3 was collected and sequenced by automated Edman degradation (Table I). A single clear sequence was evident (contamination was at most 10%, considerably less for most turns), indicating the presence of a major peptide of high purity. This allowed us to extend the sequencing through 33 turns with a high level of confidence. The sequence corresponds to that of the β subunit from residues 33 to 63. According to the published sequence of the β subunit [see Walker et al. (1985)], position 32 is an asparagine residue not an aspartate residue. We recently[2] resequenced the N-terminal portion of CF_1 β-DNA and found an aspartate residue at this position. Our peptide sequence has confirmed this result. An unknown peak with a retention time of 26.5 min (close to isoleucine) appeared in turn 31 and was assumed to be a CPM derivative of cysteine-63. There were no other significant unknown peaks, despite the low response of lysine-39 in turn 7 and the lack of a response in turn 20 (arginine-52). For reasons that we do not understand, the V8 protease failed to cleave at the aspartate residue at position 53.

Energy Transfer Measurements. The CPM derivative of cysteine-63 exhibited an unusually high quantum yield (Table II), indicating that the probe was located in a very hydrophobic

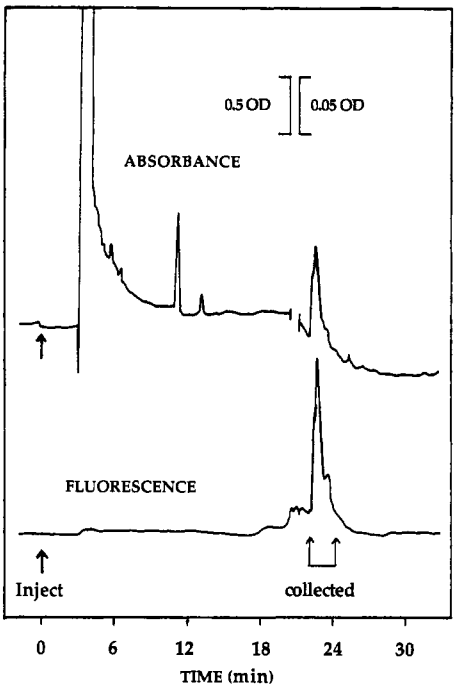

FIGURE 3: High-pressure liquid chromatography of endoprotease GluC fragments of CPM-labeled β. Proteolytic fragments of CPM-labeled β subunit were separated on a Beckman Ultrasphere ODS (25-cm, 5-μm) column using a Beckman gradient liquid chromatograph equipped with a model 166 variable-wavelength UV detector and a Model 157 fluorescence detector. System 1: Peptides were eluted with 45% (v/v) solvent A (10 mM acetic acid in water adjusted to pH 6 with triethylamine) and 55% solvent B (10 mM acetic acid in acetonitrile adjusted to pH 6 with triethylamine) for 10 min. The concentration of solvent B was changed to 65% (v/v) B in A over 3 min and elution continued. Eluate containing the fluorescent material was freeze-dried and the residue solubilized in 8 M guanidine hydrochloride for the next step. System 2: Peptides were eluted with a linear gradient of 2–60% (v/v) solvent B (0.1% trifluoroacetic acid plus 2% H_2O in acetonitrile) in solvent A (0.1% trifluoroacetic acid plus 2% acetonitrile in H_2O) over a period of 90 min. The flow rate was 1 mL/min. The elution profile for system 2 only is shown. The fluorescent material was collected as indicated, dried under nitrogen, and subjected to amino acid analysis.

environment. The observed steady-state polarization was similar to that measured previously for CPM attached to other sites on CF_1 (Richter et al., 1985). A brief exposure of the labeled β subunit to trypsin resulted in an 80% decrease in fluorescence as the probe became accessible to water, plus a decrease in steady-state polarization to a new value of 0.1, as expected. The very large change in fluorescence yield resulting from the change from hydrophobic to hydrophilic environments suggested that some of the observed fluorescence quenching upon addition of TNP-ADP (see Figure 5) may have been a consequence of a change in the local environment of the probe rather than energy transfer to TNP-ADP. To test this, we examined the effect of added ATP on CPM-β fluorescence, since ATP was shown previously to compete with TNP-ADP for binding to the same site on the β subunit (Mills & Richter, 1991). It was surprising, however, to find that the fluorescence yield of β-CPM actually increased by 4–5% upon ATP binding. Thus, ATP appeared to induce a conformational change in the β subunit which affected the environment (most likely

Table II: Energy Transfer Parameters for the CF_1 β Subunit

donor	fluorescence max (nm)	Q_D	ρ^a	acceptor	absorbance max (nm)	R_0 (Å)[b]
CPM	457	0.89	0.32	TNP-ADP	410/475	46.2

[a] Steady-state polarization measured at the fluorescence excitation and emission maxima. [b] Calculated with eq 3 using the spectral properties of the donor and acceptor shown in Figure 4. The value of κ^2 was assumed to be $^2/_3$ (Richter et al., 1985).

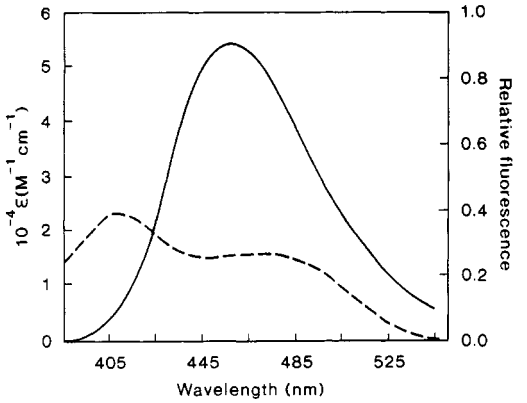

FIGURE 4: Spectral overlap of CPM and TNP-ADP. The solid line is the fluorescence emission (excitation, 387 nm) of CPM covalently attached to the β subunit. The dashed line is the extinction coefficient (ϵ) for TNP-ADP bound at the nucleotide binding site of the β subunit. Both spectra were recorded in 20 mM Tricine-NaOH (pH 8.0) at 23 °C.

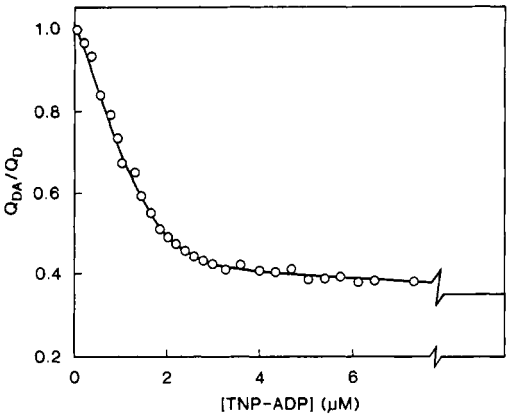

FIGURE 5: Titration of CPM-β with TNP-ADP. Q_{DA} and Q_D are the quantum yields of CPM-β in the presence and absence of TNP-ADP, respectively (excitation, 387 nm; emission, 465 nm). The titration was performed in 20 mM Tricine–NaOH (pH 8.0) at 23 °C. The end points of the titration were calculated from a Scatchard plot of the data assuming a binding stoichiometry of 1.[2]

a decrease in solvent accessibility) of the β-CPM site. The effect did not occur when nucleotide binding was destroyed either by heat treatment (Mills & Richter, 1991) or by trypsin digestion of the β subunit (results not shown). It was clear from this experiment, however, that all of the observed fluorescence quenching resulted from energy transfer from CPM to bound TNP-ADP.

The distance between CPM at cysteine-63 and the nucleotide binding site was measured by following the decrease in CPM fluorescence as a function of TNP-ADP binding. The R_0 value for energy transfer between β–CPM and TNP-ADP was calculated using the spectral overlap integral obtained from the data of Figure 4 (see Table II). The value of 46.2 Å is very similar to that calculated previously for CPM bound to sulfhydryls on the γ subunit of CF_1 (Richter et al., 1985). The results of titration of β-CPM with TNP-ADP are shown in Figure 5.

The CPM-labeled β was titrated with TNP-ADP, and TNP fluorescence enhancement was used to calculate the number of moles of TNP-ADP bound at saturation (Mills & Richter, 1991). For the experiment reported in Figure 5, this value was unity, obviating the need to correct for acceptor concentration. The maximum energy transfer efficiency was calculated from a Scatchard plot of the data of Figure 5 to give a value of 0.65. Inserting this value into eq 2 gave a distance of 41.8 Å between the two sites. A dissociation constant of 1.1 μM for TNP-ADP binding to the β subunit was also calculated from the data of Figure 5. This value is essentially identical to that reported earlier (Mills & Richter, 1991) for TNP-ADP binding to the β subunit, and is very close to the value of 1.9 μM reported for TNP-ADP binding to site 3 of CF_1 (Richter et al., 1985).

Discussion

Our results show that cysteine-63 of β is accessible to modifying reagents in isolated β but not in the β subunits associated with CF_1, or the $\alpha_3\beta_3\gamma$ complex. This suggests that cysteine-63 of each of the three β subunits is normally buried at an interface with an α subunit. An alternative explanation in that a binding interaction between the β and α/γ subunits may induce a conformational change in the β subunit such that cysteine-63 is no longer accessible to the medium. This possibility cannot be ruled out at this time.

If secondary structure predictions for the β subunit (Walker et al., 1984) are correct, then by analogy, cysteine-63 would be the 14th residue in a 20-residue α helix near the N-terminus of the CF_1 β subunit. A preliminary structural model of part of the β subunit of *Escherichia coli* F_1 from residue 141 to residue 321, which includes a putative nucleotide binding domain, was proposed by Duncan et al. (1986). Those authors suggested that the first 140 residues of the N-terminus, and the last 100 or so residues of the C-terminus of the F_1 β subunit, are not directly involved in nucleotide binding but instead form discrete domains which are important for intersubunit interactions. Our results are consistent with this hypothesis.

Current models of the F_1 ATPases [see Bianchet et al. (1991)] also suggest that there are two regions of each β subunit which are in contact with an α subunit. Several mutations at different points in the C-terminal region of the α subunit of *E. coli* F_1 have resulted in the loss of cooperativity in the enzyme, without loss of catalytic competency at individual catalytic sites (Maggio et al., 1988). On the basis of these results, it was suggested that the C-terminal region of each α subunit is in contact with a β subunit and that this interaction is involved in the signal transduction process. Our results have hinted at the existence of an ATP-induced conformational change occurring in the N-terminal region of the β subunit. It is thus possible that the catalytic sites communicate with each other via the N-terminal region of β subunits and the C-terminal region of α subunits. Although this hypothesis is very speculative at this time, it provides the basis for further experiments.

We have recently cloned and overexpressed a functional CF_1 β subunit in *E. coli*,[2] and can now place, through site-specific amino acid substitutions, potential labeling sites at strategic positions on the β subunit for attachment of fluorescent probes and bifunctional cross-linking reagents. This should allow us to test the three-dimensional structural model of the nucleotide binding domain of β (Duncan et al., 1986) and to examine further (McCarty & Hammes, 1987) the structural organization of the whole enzyme through reconstitution studies, structural mapping, and cross-linking within the CF_1 complex.

Registry No. Cys, 52-90-4; ATP, 56-65-5; ATP synthase, 37205-63-3.

References

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

Bianchet, M., Xavier, Y., Hullihen, J., Pedersen, P. L., & Amzel, M. (1991) *J. Biol. Chem. 266*, 21197–21201.

Binder, A., Jagendorf, A., & Ngo, E. (1978) *J. Biol. Chem. 253*, 3094–3100.
Boyer, P. D. (1987) *Biochemistry 26*, 8503–8507.
Boyer, P. D., & Kohlbrenner, W. E. (1981) in *Energy Coupling in Photosynthesis* (Selman, B. R., & Selman-Reiman, S., Eds.) pp 231–240, Elsevier/North-Holland, New York.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.
Bruist, M. F., & Hammes, G. G. (1981) *Biochemistry 20*, 6298–6305.
Cerione, R. A., & Hammes, G. G. (1982) *Biochemistry 21*, 745–752.
Duncan, T. M., Parsonage, D., & Senior, A. E. (1986) *FEBS Lett. 208*, 1–6.
Forster, T. (1959) *Discuss. Faraday Soc. 27*, 7–17.
Houmard, J., & Drapeau, G. R. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3506–3509.
Leckband, D., & Hammes, G. G. (1987) *Biochemistry 26*, 2306–2312.
Lien, S., & Racker, E. (1971) *Methods Enzymol. 23a*, 547–555.
Maggio, M. B., Pagan, J., Parsonage, D., Hatch, L., & Senior, A. E. (1987) *J. Biol. Chem. 262*, 8981–8984.
McCarty, R. E., & Moroney, J. V. (1985) in *The Enzymes of Biological Membranes* (Martonosi, A., Ed.) 2nd ed., pp 383–413, Plenum Press, New York.
McCarty, R. E., & Hammes, G. G. (1987) *Trends Biochem. Sci. 12*, 234–237.
Mills, D. A., & Richter, M. L. (1991) *J. Biol. Chem. 266*, 7440–7444.
Nalin, C. M., & Nelson, N. (1987) *Curr. Top. Bioenerg. 15*, 273–294.
Parker, C. A., & Reese, W. T. (1966) *Analyst (London) 85*, 587–600.
Parvari, R., Pecht, I., & Soreq, H. (1983) *Anal. Biochem. 133*, 450–455.
Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891–2899.
Penefsky, H. S., & Cross, R. L., (1990) *Adv. Enzymol. Relat. Areas Mol. Biol. 64*, 173–214.
Richter, M. L., & McCarty, R. E. (1987) *J. Biol. Chem. 262*, 15037–15040.
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.
Richter, M. L., Gromet-Elhanan, Z., & McCarty, R. E. (1986) *J. Biol. Chem. 261*, 12109–12113.
Scott, T. G., Spencer, R. D., Leonard, M. J., & Weber, G. (1970) *J. Am. Chem. Soc. 92*, 687–695.
Shapiro, A. B., & McCarty, R. E. (1988) *J. Biol. Chem. 263*, 14160–14165.
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.
Walker, J. E., Saraste, M., & Gay, N. J. (1984) *Biochim. Biophys. Acta 768*, 164–200.
Walker, J. E., Fearnley, I. M., Gay, N. J., Gibson, B. W., Northrop, F. D., Powell, S. J., Runswick, M., Saraste, M., & Tybulewicz, V. L. T. (1985) *J. Mol. Biol. 184*, 677–701.
Xue, Z., Zhou, J.-M., Melese, T., Cross, R. L., & Boyer, P. D. (1987) *Biochemistry 26*, 3749–3753.

Colchicine Photosensitizes Covalent Tubulin Dimerization

J. Wolff,* Jean Hwang, Dan L. Sackett, and Leslie Knipling
Laboratory of Biochemical Pharmacology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892

Received November 13, 1991

ABSTRACT: Pure rat brain tubulin can be cross-linked by ultraviolet irradiation of tubulin–colchicine complexes at the high-wavelength maximum of colchicine to form covalent dimers > trimers > tetramers. With colchicine concentrations $\sim 3 \times 10^{-4}$ M (mole ratio to tubulin 3–12) and irradiation for 5–10 min at 95–109 mW/cm^2, the yield of dimers is 11–17% and of trimers is 4–6% of the total tubulin. The oligomers show polydispersity and anomalously high apparent molecular masses that converge toward expected values in low-density gels. Maximal dimer yields are obtained with MTC and the decreasing photosensitizing potency is MTC > colchicine > colchicide > isocolchicine > thiocolchicine. Single-ring troponoids also promote dimerization. Evidence is presented suggesting that the initial, low-affinity, binding step of colchicine and its analogues is sufficient to photosensitize tubulin dimerization.

Covalent labeling of tubulin with [^{3}H]colchicine can be accomplished by ultraviolet irradiation of the tropolone moiety of the drug at ≈353 nm (Wolff et al., 1991). The label is found primarily in the β-monomer, but some labeling of the α-subunit can be induced under mild denaturing conditions suggesting that the drug might span the α/β subunit interface. This suggested that irradiation of bound colchicine might covalently cross-link the two subunits, and we have investigated this possibility. Earlier studies, employing various chemical cross-linking agents, had shown that covalent tubulin oligomers could be formed in good yield. Thus, Luduena et al. (1977), using chick brain tubulin, showed that $\alpha\beta$, as well as homologous dimers, could be identified by mobility and double-isotope methods; Galella and Smith (1982) found that oligomers up to hexamers were formed from tubulin treated with various bifunctional imido esters having spacers of 5–10 Å. Subsequently, it was found that hexanedione cross-linked tubulin to dimers and larger forms (Boekelheide, 1987; Sioussat & Boekelheide, 1989).

Ultraviolet irradiation of tubulin at low wavelengths has long been known to lead to destruction of mitotic spindles, etc., which is thought to result from conformational changes in