fluorescence resonance energy transfer mapping of the fourth of six nucleotide-binding sites of chloroplast coupling factor 1*

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the chloroplast atp synthase (h+-atp synthase, cf1-f0) is a complex, multisubunit enzyme exhibiting catalytic cooperativity and regulatory and catalytic conformational changes (refs. 1–4). the enzyme transduces a chemiosmotic proton gradient across the thylakoid membrane, generated in the light by the photosynthetic apparatus, into chemical bond energy in the form of the β-γ-phosphoanhydride bond of atp. in the dark, when the proton gradient dissipates, atp hydrolysis by the atp synthase is prevented by several regulatory mechanisms. these include inhibitory binding of adp (5, 6), oxidation of a dithiol to a disulfide (7, 8), and altered binding of an inhibitory subunit (9). energization of the membrane reverses these processes (10–12).

cooperativity among at least two catalytic sites of cf1, the catalytic portion of the atp synthase, is well established (13). binding of the second adenine nucleotide substrate molecule increases the catalytic rate by several orders of magnitude (14). the number of catalytic sites is unknown but is often assumed to be three. the total number of nucleotide-binding sites is thought to be six (15), as in the homologous escherichia coli and mitochondrial enzymes (16). some of these sites are noncatalytic (17–19).

the number and properties of all the nucleotide-binding sites are essential information for understanding the catalytic mechanism and regulation of the chloroplast atp synthase. three of the sites were characterized by bruist and hammes (17). sites 1 (20) and 3 (17) are believed to be catalytic, whereas site 2 is noncatalytic (17). we characterized two more sites (18). our data suggested that one of these may be catalytic and the other, non-catalytic. boyer’s laboratory has also examined nucleotide binding to cf1. their data indicate one to two catalytic and three noncatalytic sites (19). they have also found evidence for a regulatory role of noncatalytic sites (21, 22).

information about the positions of the nucleotide-binding sites on cf1 could be useful for understanding their interactions. fluorescence resonance energy transfer distance measurements (23) have yielded a map for nucleotide-binding sites 1, 2, and 3, as well as additional sites not involved in nucleotide binding. in this paper, we report fluorescence energy transfer distance mapping of nucleotide-binding site 4, which may be catalytic. we also present data showing the existence of a total of six nucleotide-binding sites of cf1.

materials and methods

cf1 was prepared as described previously (24). contaminating ribulose bisphosphate carboxylase/oxygenase was removed by affinity chromatography. the cf1 was stored as an ammonium sulfate precipitate at 4 °c.

cf1 from which the δ and ε subunits had been removed (cf1-(δ,ε)) was prepared as described in ref. 25. it was stored as an ammonium sulfate precipitate at 4 °c. the δ and ε subunit-containing fractions were pooled and concentrated by ultrafiltration with an amicon pm-10 membrane. the concentrate was stored at -20 °c.

equilibrium dialysis—cf1 was desalted by passage through two consecutive sephadex g-50 (fine) centrifuge columns (28) equilibrated with 50 mm tris-hcl, ph 8, 50 mm nacl, and 2 mm edta (tne-8 buffer). the cf1 was reductively activated by incubation with 50 mm dithiothreitol overnight at 23 °c. the dithiothreitol was removed by passage of the enzyme solution through two consecutive centrifuge columns equilibrated with 50 mm tris-hcl, ph 8, and 50 mm nacl (tn-8 buffer). the reduced disulfide bond was then prevented from reoxidizing by reacting the sh groups with 5 mm n-ethylmaleimide for 10 min at 23 °c, followed by passage through two
consecutive centrifuge columns equilibrated with TN-8 buffer to remove excess N-ethylmaleimide.

Activated CF$_1$ (10 nmol) in TN-8 buffer was placed in one 100-μl half-cell of a Lucite equilibrium dialyzer (Sedereware). The nucleotide to be tested (100 nmol), also in TN-8 buffer, was placed either in the same half-cell as the CF$_1$, in the opposite side of the membrane as the CF$_1$, the concentrations of AMP-PNP, ADP, and AMP-PNH$_2$ were determined from the absorbance at 259 nm of the protein-free half-cell, using an extinction coefficient for adenine of 15,400 M$^{-1}$ cm$^{-1}$.

The free nucleotide concentration was calculated as follows. The concentration of nucleotide in the half-cell without CF$_1$ was multiplied by the volume of liquid in the whole cell (200 μl), giving the amount of nucleotide. This amount was subtracted from the initial amount of nucleotide added to the cell, as determined from the nucleotide concentration in protein-free control cells, giving the amount of bound nucleotide. This amount was divided by the amount of CF$_1$ in the cell, giving the mole of nucleotide/mole of CF$_1$. An additional 1.5 mol of nucleotide/mol of CF$_1$ was added to this figure when the test nucleotide was ADP, AMP-PNP, or AMP-PNH$_2$.

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Labeling the Lucifer Yellow Site (27)—CF$_1(-6,e)$ was desalted by passing it through two consecutive centrifuge columns equilibrated with 50 mM Bicine-NaOH, pH 9, and 3 mM MgCl$_2$. Lucifer Yellow vinyl sulfone (Aldrich) dissolved in dimethyl sulfoxide was added to the labeled CFI sample (1 mg/ml). The sample was incubated at 23 °C for 1 h, heated in boiling water for 10 s, and cooled. A second addition of trypsin was made, and the sample was incubated overnight at 23 °C. After centrifuging at 100,000 g for 5 min, 100 μl was injected into an Ultraphase ODS 4.6 × 250-mm reversed phase column attached to a Beckman 342 gradient chromatograph equipped with a model 160 absorbance detector set at 214 nm, and a fluorescence detector attached to a Spectraphysics SP4270 integrator. The flow rate was 2 ml/min. Lucifer Yellow-labeled CF$_1(-6,e)$ the gradient was from 2 to 45% (v/v) acetonitrile in water for 60 min. For CPM-labeled CF$_1(-6,e)$ the gradient was from 20-45% (v/v) acetonitrile in water for 60 min. For eosin maleimide-labeled CF$_1(-6,e)$ the 2-45% gradient was used. Trifluoroacetic acid was present at 0.1% (v/v) in both water and acetone.

Subunit specificity of labeling was also checked by examining the fluorescent protein bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Fluorescent bands were visualized by placing the unstained gel on an ultraviolet transilluminator. Water was used to label the γ subunit, a small amount of labeling occurred on the α and β subunits. CPM labeling of the ε subunit was completely
specific. Lucifer Yellow labeled the δ and γ subunits slightly. Eosin maleimide was completely specific for the γ subunit.

Fluorescence Measurements—Steady-state fluorescence was measured with a Shimadzu RF-5000U spectrofluorometer. For fluorescence resonance energy transfer measurements, the energy transfer efficiency was taken as the quotient of the fluorescence of a sample containing donor and acceptor probes divided by the fluorescence of a sample containing only the donor probe. Samples were diluted sufficiently to make the inner filter effect negligible, usually about 0.5 μM protein. Correction was made for differences in protein concentration between samples. Protein concentration was measured by the method of Lowry et al. (35). The molecular weights of CF,(δ,ε) and CF, are 364,000 and 400,000 (36), respectively.

The corrected fluorescence emission spectrum of Lucifer Yellow was measured and integrated with an SLM Aminco SPF-5000C spectrofluorometer. The absorbance spectrum of eosin maleimide was taken with a Shimadzu UV160U spectrophotometer. The overlap integral for the Lucifer Yellow-eosin maleimide pair (Fig. 1) was calculated according to Ref. 37. The quantum yield of Lucifer Yellow attached to CF, was taken as 0.36 (27). The TNE-8 buffer had a refractive index of 1.335. The absorbance of eosin maleimide was completely specific for the γ subunit. Eosin maleimide was used with a Shimadzu RF-5000U spectrofluorometer. The overlap of Lucifer Yellow fluorescence emission and eosin maleimide absorbance was calculated according to Ref. 37 to be 52.8 Å, assuming a value of 2/3 for K2.

Values of R0 for the Lucifer Yellow-TNP-ATP and CPM-TNP-ATP pairs for each donor site were taken from the literature. For Lucifer Yellow and TNP-ATP in site 1, the R0 is 34.8 Å (37). For CPM and TNP-ATP in site 1, the R0 for the γ subunit disulfide bond sulfhydryls is 45.2 Å (31), for Cys-322 of the γ subunit is 45.6 Å (30), and for the ε sulfhydryl is 44.8 Å (9). TNP-ATP in site 4 was assumed to give the same R0 values as TNP-ATP in site 1.

Distances between donor and acceptor were calculated from the R0 and energy transfer efficiency according to Ref. 37.

Acceptor stoichiometries (TNP-ATP and eosin maleimide) were calculated by subtracting the absorbance of the protein bearing only the donor from the absorbance of the protein bearing both the donor and acceptor at the absorption maximum of the acceptor and correcting for differences in protein concentration. The corrected acceptor absorbance was divided by the extinction coefficient for the bound acceptor to get the acceptor concentration, which was then divided by the protein concentration to get the acceptor/CF, stoichiometry.

Donor/CF, stoichiometries were not calculated, but conditions were chosen to yield stoichiometries less than 0.4 mol of donor/mol of CF, in order to maintain high labeling specificity.

RESULTS

Equilibrium Dialysis—There are believed to be six nucleotide-binding sites on CF, as in mitochondrial and bacterial

![Fig. 1. Spectral overlap of Lucifer Yellow fluorescence emission and eosin maleimide absorbance.](image)

Table 1

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>mole nucleotide bound/mole CF,</th>
<th>Total mole nucleotide bound/mole CF,</th>
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<tr>
<td>Mg&quot;-AMP-PNP</td>
<td>3.9 ± 0.3</td>
<td>5.4</td>
</tr>
<tr>
<td>AMP-PNP</td>
<td>1.0 ± 0.3</td>
<td>2.5</td>
</tr>
<tr>
<td>Mg&quot;-ADP</td>
<td>4.9 ± 0.4</td>
<td>6.4</td>
</tr>
<tr>
<td>ADP</td>
<td>2.5 ± 0.2</td>
<td>4.0</td>
</tr>
<tr>
<td>Mg&quot;-AMP-PNH2</td>
<td>2.1 ± 0.5</td>
<td>3.6</td>
</tr>
<tr>
<td>Mg&quot;-TNP-ATP</td>
<td>7.0 ± 0.9</td>
<td>7.5</td>
</tr>
<tr>
<td>Mg&quot;-TNP-ADP</td>
<td>6.3 ± 0.1</td>
<td>6.8</td>
</tr>
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</table>

Equilibrium dialysis experiments

The calculations are described under “Materials and Methods.” Total mole of nucleotide bound/mole of CF, includes an additional 1.5 mole of endogenous ADP/mole of CF, for the experiments with AMP-PNP, AMP, and AMP-PNH2 and an additional 0.5 mole of endogenous ADP/mole of CF, for the experiments with TNP-ADP and TNP-ATP added to the mean mole of nucleotide bound/mole of CF,. Values are means ± S.D.; n = 4.

Non-equilibrium binding measurements have revealed the presence of four sites which bind nucleotides tightly under appropriate conditions (18, 19). These sites are labeled 1, 2, 4, and 5 (17, 18). Photoaffinity labeling studies showed that each of the three β subunits of CF, can bind two nucleotides (19). Using the Hummel and Dreyer gel filtration method, Girault et al. (15) found that approximately six nucleotides bound to CF, under equilibrium conditions. Those experiments were restricted to nucleotide concentrations less than 150 μM so that the ultraviolet absorbance of the nucleotides did not saturate the detector, and so that a difference due to nucleotide binding to CF, (typically 0.6 nmol) could be observed. Here we show a similar result from equilibrium dialysis experiments. We used high concentrations of nucleotides and enzyme in order to saturate all available nucleotide-binding sites with dissociation constants less than a few hundred micromolar. We also showed that complete saturation requires a divalent cation. The results are shown in Table 1.

Equilibrium dialysis with ATP was not performed because the ATP would have been rapidly hydrolyzed to ADP. AMP-PNP is a non-hydrolyzable analog of ATP which binds in the same fashion as ATP (18). AMP-PNP (ICN Biochemicals) + endogenous ADP filled nearly six sites when 100 nmol of AMP-PNP was incubated with 10 nmol of CF, in the presence of 50 nmol of MgCl2. If the MgCl2 was replaced with 200 nmol of EDTA, binding was much reduced. Some spontaneous degradation of AMP-PNP to AMP-PNH2 occurs during prolonged incubation at room temperature. This may reduce the amount of bound nucleotide, since AMP-PNH2 does not bind well to CF, (18). We found previously that CF, does not have any sites which bind tightly to Mg"-AMP-PNH2, whereas it has four tight binding sites for Mg"-AMP-PNP and two for Mg"-ADP. A tight binding site is defined here as one which retains bound nucleotide when the CF, is passed through Sephadex G-50 centrifuge columns. As expected, Mg"-AMP-PNH2 fills only 2.1–3.6 sites during equilibrium dialysis (Table 1). We showed previously that Mg"-AMP-PNH2 does not displace any of the tightly bound, endogenous ADP (18), so only 2.1 sites/CF, were occupied by Mg"-AMP-PNH2 under equilibrium dialysis conditions.

CF, has only two tight binding sites for ADP or Mg"-ADP (18). Under equilibrium dialysis conditions, however, CF, binds 6 mol of Mg"-ADP/mol of CF,. In the absence of Mg"+, only 4 mol of ADP bind/mol of CF,. Interestingly, Mg"+ does not influence the number of tight ADP-binding sites on CF,, whereas Mg"+ increases the number of tight AMP-PNP- and
ATP-binding sites from two to four (including unexchanged endogenous ADP) (18).

The ATPase activity of the CF$_1$ samples in the equilibrium dialysis experiments was not measured. Therefore, the effect of the nucleotide and Mg$^{2+}$ incubations on the ATPase activity was not determined.

Equilibrium binding of the 2′-(3′)-O,2,4,6-trinitrophenyl analogs of ADP and ATP was tested. TNP-ATP is a useful analog for fluorescence resonance energy transfer distance measurements in which it is used as an acceptor. Because its concentration can be measured by its absorbance at 408 nm, the amount bound to CF$_1$ can be determined without interference from endogenous ADP. In the presence of Mg$^{2+}$, 6–7 mol of TNP-ATP or TNP-ADP bound/mol of CF$_1$. In addition, up to about 0.5 mol of endogenous ADP/mol of CF$_1$ may remain bound. After 2 h at 23 °C with 3 mM TNP-ATP and 1 mM MgCl$_2$, reductively activated CF$_1$ retained 0.5 mol of endogenous ADP/mol of CF$_1$ (not shown).

Fluorescence Resonance Energy Transfer Distance Measurements—The relative distances between the nucleotide-binding sites could be useful information for understanding their interactions. In particular, nearby sites or sites located on the same α/β pair may be more likely to interact than distant sites or those located on different α/β pairs. Furthermore, the locations of the nucleotide-binding sites with respect to regulatory regions of CF$_1$, such as the γ and ε subunits, could suggest regulatory mechanisms. Therefore, we undertook fluorescence resonance energy transfer distance mapping of one of the newly characterized tight nucleotide-binding sites, site 4.

Site 4, like site 1, binds ADP and ATP tightly, does not require Mg$^{2+}$ for tight binding, and exchanges with medium nucleotides. Because of the similarity between the properties of site 4 with those of site 1, and because of our data showing ATP hydrolysis in site 4 in the absence of medium nucleotides (18), we suggested that site 4 may be catalytic. If so, it probably interacts cooperatively with one or more other nucleotide-binding sites.

Incubation of CF$_1$ with low concentrations of TNP-ATP, followed by removal of excess and loosely bound nucleotide by gel filtration, results in incorporation of TNP-ATP into site 1. Site 2 will also be filled by TNP-ATP if Mg$^{2+}$ is included. At higher TNP-ATP concentrations, in the absence of Mg$^{2+}$, the TNP-ATP/CF$_1$ stoichiometry approaches 2.0, suggesting that both sites 1 and 4 are filling. Saturation requires about 3 mM TNP-ATP. This is the nominal concentration. Samples of TNP-ATP usually consist of a mixture of TNP-ATP and TNP-ADP. Both bind to sites 1 and 4.) The concentration of TNP-ATP required for saturation of sites 1 and 4 is about the same as the concentration of ADP, ATP, or AMP-PNP required to saturate tight binding sites (18). Some endogenous ADP does not exchange with TNP-ATP, so complete saturation of sites 1 and 4 with TNP-ATP is not possible. This was also observed with Mg$^{2+}$-AMP-PNP (18).

TNP-ATP is a useful acceptor probe for fluorescence energy transfer. Although site 4 alone can not be filled with TNP-ATP, distances from various donor sites to site 4 can still be derived from experiments in which TNP-ATP inhabits sites 1 and 4. First, the distance between the donor site and site 1 must be known. Second, site 1 must be saturated with TNP-ATP and the stoichiometry of TNP-ATP filling of site 4 must be known. The first condition is easily met because abundant distance information is available for site 1 (9, 27, 30, 31, 38). To meet the second condition we incorporated TNP-ATP to several stoichiometries and approached saturation as closely as possible, then extrapolated the energy transfer efficiency

versus TNP-ATP/CF$_1$, curve to a stoichiometry of 2.0.

It was not feasible to use other nucleotide-binding sites as donor sites for energy transfer to site 4 because it was not possible to assure site specificity. The donor sites used were (i) the γ subunit disulfide bond sulphydryls (Cys-199 and Cys-205), (ii) the γ subunit Cys-322 sulphydryl, (iii) the ε subunit sulphydryl (Cys-6), and (iv) the Lucifer Yellow vinyl sulfone labeling site (Lys-378) present on only one of the three α subunits. For the sulphydryl sites, CPM was the donor probe. For each site, the R$_0$, the distance between donor and acceptor at which the energy transfer efficiency is 0.5, has already been reported (9, 27, 30, 31, 38).

For each donor site labeling reaction, specificity of the covalent reaction was tested by high pressure liquid chromatography of complete trypsin digests of the labeled protein. Representative fluorescence elution profiles are shown in Fig. 2. For the γ disulfide-labeled CF$_1$, the two peaks represent labeling of the 2 cysteine residues. For the ε sulphydryl-labeled CF$_1$, the double peak probably represents oxidation of the NH$_2$-terminal methionine residue, since the relative sizes of the two peaks changed with the age of the labeled protein (not shown). The CPM probe appears to contain three isomers with slightly different mobilities on HPLC, as judged by the elution profile of CPM-labeled N-acetyl-L-cysteine (not shown). This produces the frequently observed separated or merged triplet peaks.

The energy transfer efficiency versus TNP-ATP/CF$_1$, stoichiometry graphs are shown in Fig. 3. It is clear from Fig. 3A that site 1, which is very far from the disulfide bond (31), begins filling with TNP-ATP first, as indicated by the small slope of the curve at low TNP-ATP/CF$_1$, stoichiometry. Site 4 begins filling after site 1 has filled to a stoichiometry of about 0.5 TNP-ATP/CF$_1$. Site 1 is completely filled with TNP-ATP by a stoichiometry of about 1.5 TNP-ATP/CF$_1$, as indicated by the end of the curved portion of the graph and the start of the steep linear portion. Linear extrapolation of the data for TNP-ATP/CF$_1$, stoichiometries higher than 1.5 to a stoichiometry of 2.0 yields the energy transfer efficiency when both sites 1 and 4 are saturated with TNP-ATP.

Fig. 2. Representative HPLC fluorescence elution profiles of trypsin-digested CF$_1$(-4α) or CF$_1$, labeled at each donor site. A, CF$_1$(-4α) labeled with CPM at the γ subunit disulfide bond sulphydryls (Cys-199 and Cys-205). B, CF$_1$(-4α) labeled with CPM at Cys-322 of the γ subunit. C, CF$_1$(-4α) labeled with Lucifer Yellow VS at Lys-378 of a single a subunit. D, CF$_1$(-4α) reconstituted with δ subunit and ε subunit labeled with CPM at Cys-6. E, CF$_1$(-4α) labeled with Lucifer Yellow VS at Lys-378 of a single a subunit and eosin maleimide at the γ subunit disulfide bond sulphydryls. Fluorescence is in arbitrary units.

2 A. B. Shapiro and R. E. McCarty, unpublished result.
Mapping Nucleotide-binding Site 4 of CF₁

**Fig. 3.** Energy transfer efficiency as a function of TNP-ATP acceptor/CF₁(-δ,ε) stoichiometry. A, CF₁(-δ,ε) labeled with CPM at the γ subunit disulfide bond sulfhydryls (Cys-199 and Cys-205). B, CF₁(-δ,ε) labeled with CPM at Cys-322 of the γ subunit. C, CF₁(-δ,ε) (○) and CF₁ labeled with Lucifer Yellow VS at Lys-378 of a single α subunit (△). D, CF₁(-δ,ε) reconstituted with δ subunit and ε subunit labeled with CPM at Cys-6.

**Fig. 4.** Energy transfer efficiency between Lucifer Yellow VS at Lys-378 of a single α subunit and eosin maleimide at the γ subunit disulfide bond sulfhydryls (Cys-199 and Cys-205) attached to CF₁(-δ,ε) as a function of eosin maleimide acceptor/CF₁(-δ,ε) stoichiometry.

Two slopes are also evident in each of the other curves in Fig. 3. In Fig. 3B, site 1 is closer to the donor probe at Cys-322 of the γ subunit than site 4. The slope at low TNP-ATP/CF₁ stoichiometry is high, whereas the slope at high TNP-ATP/CF₁ stoichiometry is low. In Fig. 3D, the slopes in the two regions are similar, but the slope representing the distance from Cys-6 of the ε subunit to site 4 is slightly larger than the slope representing the distance to site 1. Thus site 4 is closer to Cys-6 than is site 1. The only exception to this pattern is the result for the Lucifer Yellow site (Fig. 3C), for which it appears that the majority of site 4 filling occurred between TNP-ATP stoichiometries of 0.5 and 1.0, beyond which additional filling of distant site 1 occurred. This result may be a statistical artifact produced by the difficulty of measuring very low energy transfer efficiencies. Fortunately, the distance measurement is not very sensitive to small errors in the extrapolated energy transfer efficiency.

Fig. 3C shows that there is no difference, within experimental error, in the Lucifer Yellow to TNP-ATP energy transfer efficiencies between CF₁ and CF₁(-δ,ε), indicating that removal of the δ and ε subunits does not greatly alter the shape of the remainder of the enzyme. Mitra and Hammes (29) showed that removal of the δ and ε subunits has no measurable effect on the distances between Cys-322 on the γ subunit and disulfide bond and between Cys-322 and nucleotide-binding sites 1, 2, and 3.

The extrapolated energy transfer efficiencies at a TNP-ATP/CF₁ stoichiometry of 2.0 from Fig. 3 are 0.54 (Fig. 3A), 0.46 (Fig. 3B), 0.13 (Fig. 3C), and 0.26 (Fig. 3D).

To derive the distances between site 4 and the donor sites when both sites 1 and 4 are filled with acceptor TNP-ATP, the distances between site 1 and the donor sites must be known. The measurements were made previously (9, 27, 30, 31, 38), but in some cases the distances were so great that only a lower limit could be determined. Modeling was done to determine the distances from the available measurements.
A model for CF1 was generated from all the distance measurements made prior to this paper for the sites within the CF1 molecule, using algorithm for least-squares optimization described in the Miniprint. The computations yielded two equally likely solutions, which were nearly identical except for the position of the subunit disulfide bond. This occurred because the site had an insufficient number of well-defined distances measured between it and other sites.

This problem was approached by making an additional measurement involving the disulfide site. A previous attempt to measure the disulfide bond-Lucifer Yellow site distance had yielded only a lower limit (27) because no donor-acceptor pair was available with a sufficiently large . We solved this problem by using eosin maleimide as an acceptor for Lucifer Yellow fluorescence. The for this pair was an exceptionally large 52.8 Å, thanks to an excellent spectral overlap, a high quantum yield for Lucifer Yellow fluorescence and an extremely high extinction coefficient for eosin maleimide.

The energy transfer efficiency at an eosin maleimide stoichiometry of 1.0 was calculated to be 0.21 from a linear regression fit to the energy transfer versus eosin maleimide/CF1 stoichiometry plot (Fig. 4). This efficiency corresponds to a distance of 66 Å.

After adding the Lucifer Yellow-disulfide bond distance to the other distance measurements, the least-squares optimization yielded a single model. The best-fit distances between the four donor sites and site 1 (73.0 Å for the subunit disulfide bond sulphydryl, 48.6 Å for Cys-322 of the subunit, 63.8 Å for the Lucifer Yellow site, and 62.7 Å for the subunit sulphydryl) were used to calculate the distances between the donor sites and site 4 (Table 2). All of the distance measurements including the new ones involving site 4 were used to generate new least-squares best-fit models. A single model resulted. All measured distances, and the best-fit distances computed from the model, are shown in Table 2. Despite significant uncertainties in the energy transfer measurements, in particular the true value of and the often large scatter in the data, no distance in the final model deviated by more than 7.5% from the measured distance, only six distances deviated by more than 4%, and only one distance bound was violated.

A set of Cartesian coordinates showing the relative positions of the nine energy transfer sites in the final model is presented in Table 3.

**DISCUSSION**

The equilibrium dialysis experiments confirm the results of (15) that CF1 contains six nucleotide-binding sites. In addition, the experiment shows that the divalent cation Mg++ substantially increases the binding affinities of some of the other sites.

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<tr>
<th>N1</th>
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3) Portions of this paper (including part of "Discussion" and Table 2) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
Mapping Nucleotide-binding Site 4 of CF₁

A search was made for additional orientations of the energy transfer model to fit the electron microscope image. Two criteria in addition to the above were applied. First, the γ subunit sulfhydryls must be at similar distances from the thylakoid membrane as in the orientation in Fig. 6A to agree with the measured distances of closest approach (31, 39). Note that some flexibility is possible since these distances need not be perpendicular to the plane of the membrane; the protein chains of the ATP synthase may exclude membrane lipid from directly beneath the γ sulfhydryls.

Second, the four mapped nucleotide-binding sites must lie in at most two planes parallel to, or nearly parallel to, the plane of the membrane. We assume that the pseudo 3-fold symmetry axis of CF₁ is normally oriented perpendicularly to the thylakoid membrane surface. Then the distance above the membrane of a nucleotide binding site on one β subunit or at one α/β interface is on when the sites are formed from the same amino acid residues. Since each α/β pair contains two nucleotide-binding sites (19) consisting of different amino acid residues, the six sites must occupy at most two planes. Assuming no major distortion of the CF₁ molecule due to the presence of the single copy subunits, both planes should be parallel to the thylakoid membrane surface.

The orientation of the energy transfer model shown in Fig. 6A fits the above criteria. Sites 2 and 3 lie in one plane parallel to the membrane surface and sites 1 and 4 lie in another. Sites 1 and 4 are closer to the membrane than sites 2 and 3. The γ subunit sulfhydryls are at appropriate distances from the membrane. The view from above the membrane is shown in Fig. 6B. This orientation may be superimposed upon the electron microscope image as well as the orientation shown in Fig. 5B, using the several criteria stated above, except that the ε sulfhydryl falls in the area identified as containing the δ subunit rather than the nearby area containing the ε subunit (40). This is not a serious flaw, however, because it is possible that part of the ε subunit is in the δ subunit area.

For both orientations, when the Cys-322 and Cys-89 of the γ subunit are placed on or near the central γ subunit density, all four nucleotide-binding sites may be placed on β subunits or at α/β interfaces. The ε subunit sulfhydryl and γ subunit disulfide bond may also be placed in appropriate positions. The Lucifer Yellow site does not fall on the main density of an α subunit, however. Instead, it is on the edge of an α subunit area. The nearby γ subunit provides the unique environment that lowers the pKc of the ε-amino group of this partic-

![Diagram of CF₁](image)

**Fig. 5.** Two views of the fluorescence energy transfer map of CF₁ arranged so that nucleotide binding sites 1, 2, and 3 lie in a plane parallel to thylakoid membrane surface. A, "side" view with the membrane surface perpendicular to the plane of the page. B, top view with the membrane surface in the plane of the page superimposed upon the CF₁ electron microscope average image (40). Symbols are as in Fig. 5.
ular lysyl residue. Indeed, it would be more difficult to understand how single-site labeling by Lucifer Yellow could occur if the site were on the main body of the α subunit, where it would be less likely to have a substantially different environment from one α subunit to the next.

Nucleotide-binding site lying in the same plane should occupy similar positions on their respective β subunits or at their respective α/β interfaces because the sites should consist of the same amino acid residues. Presumably, the three β subunits or α/β pairs have similar if not identical tertiary structures. Three nucleotide-binding sites sharing a plane parallel to the membrane plane should also be symmetrically arranged around the center of the CF$_1$ molecule at the vertices of an equilateral triangle, although the asymmetry of the CF$_1$ molecule may cause the triangle to be distorted. In Fig. 5B, sites 1, 2, and 3 are arranged in a small triangle near the middle of the CF$_1$ molecule. Sites 4, 5, and 6 must then form a large triangle with vertices near the periphery of the molecule. In Fig. 6B, sites 2 and 3 form the small, central triangle with site 5 or 6, whereas sites 1 and 4 form the larger triangle with site 6 or 5. Otherwise the two orientations place the nine energy transfer sites similarly within the electron microscope image. Viewed parallel to the plane of the membrane (Figs. 5A and 6A), however, there are substantial differences. Energy transfer distance measurements between the membrane surface and the nucleotide-binding sites may be able to distinguish between the two models.

Boekema$^4$ has recently obtained data to suggest that the subunit at the twelve o’clock position in the electron microscopic single particles average image is an α subunit. If that is so, then the orientation of the energy transfer map shown in Fig. 6B is more likely than the one shown in Fig. 5B.

The two orientations of the energy transfer map suggest different interactions between pairs of nucleotide-binding sites. According to the alternating site binding hypothesis of cooperative catalysis by the ATP synthase (41), two or three sites interact cooperatively, alternating nucleotide-binding properties sequentially during each catalytic cycle. At any given moment during catalysis, one nucleotide-binding site in a cooperative unit will have tight nucleotide-binding properties and another site will have loose binding properties. It has frequently been assumed, because of the 3-fold symmetry of the CF$_1$ molecule and the good fit of three-site models to kinetic data (13, 41), that three sites are involved in a single cooperative unit, with all three sites engaged in catalysis. The pairwise similarity of properties of the six nucleotide-binding sites (18) suggests, however, that two cooperative units may exist, each consisting of a tight binding, exchangeable site (sites 1 and 4) and a loose binding site (sites 3 and 6). Each unit may also be regulated by a tight binding, nonexchangeable, noncatalytic site (sites 2 and 5). Noncatalytic sites have recently been shown to regulate the rate of catalysis under certain conditions (21, 22). The energy transfer map and electron microscope image may suggest which pairs of sites are most likely to interact. Those which are located close together in space or on the same α/β pair may be more likely to interact cooperatively than those at a large distance.

One difficulty with any orientation of the energy transfer map with respect to the electron microscope image is that, for at least one of the three pairs of similar nucleotide-binding sites, one of the two sites will be in the inner triangle of sites and one will be in the outer triangle. It is reasonable to expect that the nucleotide-binding sites in the two environments consist of different amino acid residues. This difference would seem to preclude the two sites having identical properties. Note, however, that although the two sites in each pair have similar properties, they are not identical. For example, site 4 requires higher TNP-ATP concentrations than site 1 in order to bind TNP-ATP tightly. There is no a priori reason why two nucleotide-binding sites cannot have similar properties despite being comprised of different portions of the tertiary structure of the protein.

Based on the proximity criterion, the orientation of the energy transfer model shown in Fig. 5B predicts that sites 3 and 4 form one catalytic pair and sites 1 and 6 form another. The orientation shown in Fig. 6B predicts that sites 1 and 3 form one catalytic pair and sites 4 and 6 form the other.

If there really are two cooperative catalytic units, each consisting of two alternating catalytic sites and one regulatory noncatalytic site, the question arises as to whether they interact or are independent. Given the highly cooperative nature of interactions within the CF$_1$ molecule, interacting catalytic units seem likely. If so, the rate of a communicating conformational change between two cooperative catalytic units, for example, could be enhanced as compared to cooperative catalysis by CF$_1$. It would be of interest to see whether the available data (13, 41) would fit such a model as well as a three-site-binding change mechanism.

Using fluorescence resonance energy transfer between Lucifer Yellow and TNP-ATP in isolated CF$_1$, we showed previously (24, 42) that site 3 switched its properties from loose binding to tight binding upon binding Mg$^{2+}$-ATP or Mg$^{2+}$-AMP-PNP. Simultaneously, another site, distant from the Lucifer Yellow site switched from tight binding to loose binding. We concluded that site 3 was engaged in alternating site catalysis with site 1, as the properties of sites 4, 5, and 6 had not yet been characterized. The energy transfer distance map will make it possible to use a similar technique to test whether site 4 is also involved in alternating site catalysis and determine which pairs of sites interact.

Fluorescence energy transfer distance mapping, along with characterization of the properties of all six nucleotide-binding sites of CF$_1$, continue to reveal important structural and functional features of the chloroplast ATP synthase. This information will eventually contribute to a detailed understanding of the catalytic and regulatory mechanisms of this complex enzyme.

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REFERENCES

$^4$ E. J. Boekema, personal communication.
Much effort and ingenuity, both theoretical and practical, has been devoted to the problem of deducing three-dimensional structural models from experimentally measured distances between atoms on a macromolecule or macromolecular complex (for reviews see Refs. 41-46). The most important biochemical application for algorithms that have been developed for solving the problem is the deduction of protein conformations from data obtained by NMR techniques. Such measurements produce a great many short upper bounds for distances between sites; in general, these are not very precise, having errors which can easily reach 50%. The measurements also lead to bounds for many of the dihedral angles that determine the conformation of the protein. The addition of static constraints leads to a set of lower bounds which, with the upper bounds and the bounds for dihedral angles, can be used to allow such algorithms to build a set of similar conformations for the protein.

The algorithm described here is designed to solve a problem in which the experimental data have a different character. By contrast with the data from NMR, most of the data from fluorescence energy transfer measurements lead to estimates for internuclear distances that can be regarded as accurate within 3%. In addition, upper and lower bounds may be obtained for some other interatomic distances. The number of experimentally determined distances is also much smaller than in the NMR case, which greatly simplifies the computational problem.

The algorithm proceeds in two steps, by first building up a structure satisfying the distance measurements approximately, and then refining this structure using non-linear least squares. The first step finds an approximate structure, now a subset of the set of all solutions to the distance measurements, which approximates to a rigid molecule. This first step finds an approximate starting structure, which is used as a rigid structure to which the second step is applied.

Once a starting structure has been found, the second step proceeds by introducing a small change, such as a rotation or a translation of the molecule. This small change is then constrained to a new set of solution distances, which is found by minimization of a function which is the sum of squared differences between the measured distances and the distances determined from the new solution structure, taking into account the uncertainty of the measurements.

The algorithm proceeds iteratively, adding new solution structures to the set as new measurements are made, and refining the existing solution structures until a final solution is obtained.
This build-up procedure for obtaining a starting structure is effective mainly because of the rather high degree of accuracy of distances measured by fluorescence energy transfer.

Positioning new points. Let A, B, and C be three points whose positions have been verified, and let a, b, and c be the distances of a new point P from A, B, and C, respectively. Then P’ is one of the two points of intersection of three spheres centered at A, B, and C, with radii a, b, and c. Methods for computing the positions of these points, knowing the positions of the centers of the spheres and their radii, have been presented by several authors (48-50); we have chosen to use the method in Ref. 8. The distances of the two possible positions for P from a fourth point D whose position has been set are computed and compared with the measured distance from P to D. If all the distances between the points A, B, C, D, and P were exact, one of these computed distances would be exactly equal to the measured distance; however, because of experimental error this is highly improbable. In the present study, we have found the following criteria for choosing between the two possible points to be satisfactory: (i) if the two computed distances are almost equal within 0.1 Å, P is placed halfway between the two possible points; (ii) if one distance is less than the other and its difference from the measured distance does not exceed 10 Å, it is used to define the position of P. If no point D can be found for which either (i) or (ii) is satisfied, the two positions for P are examined to determine whether one of them satisfies all measured upper and lower bounds for distances that have been set previously, while the other does not. If this test also fails, the point is considered to be ambiguous.

Structure refinement. The approximate structure is refined by minimization of the least-squares function:

\[
F = \sum_{i=1}^{N} \left( y_{i} - \left[ x_{i} - x_{i}^{a} \right] \right)^{2} + \sum_{i=1}^{N} \left( y_{i} - \left[ y_{i} - y_{i}^{a} \right] \right)^{2} + \sum_{i=1}^{N} \left( z_{i} - \left[ z_{i} - z_{i}^{a} \right] \right)^{2}
\]

Here \( x_i \) is the coordinate vector of the \( i \)-th site, \( d_{ij} \) is the measured distance between the \( j \)-th and \( k \)-th sites, \( u_{ij} \) is an upper bound for the distance between the \( k \)-th and \( l \)-th sites, and \( l_{ij} \) is a lower bound for the distance between the \( i \)-th and \( j \)-th sites. The first term is summed over all pairs of points for which the distances have been measured, the second term is summed over all pairs of points for which an upper bound to the distance is known, and the third term is summed over all pairs of points for which a lower bound to the distance is known. The symbol \( M \), indicates that the term in parentheses takes only positive or zero values. For N points, at least 4N-10 distances must be known if the structure is to be determined (47, 51). Since there are only 3N-6 independent variables, the number of redundant parameters exceeds the number of variables and the problem is well-posed. The independent variables are allotted as follows: the first point is taken to be fixed, the second point has a variable \( x \)-coordinate but fixed \( y \)- and \( z \)-coordinates, and the third point has variable \( x \)- and \( y \)-coordinates but a fixed \( z \)-coordinate. All other points have three variable Cartesian coordinates.

**Table 3. Cartesian coordinates for the nine fluorescence energy transfer sites, derived from the distance measurements by least-squares optimization.**

<table>
<thead>
<tr>
<th>Site</th>
<th>( x_{i} )</th>
<th>( y_{i} )</th>
<th>( z_{i} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>0.0</td>
<td>0.0</td>
<td>3.0</td>
</tr>
<tr>
<td>N2</td>
<td>39.7</td>
<td>30.5</td>
<td>0.0</td>
</tr>
<tr>
<td>N3</td>
<td>48.4</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>D</td>
<td>28.5</td>
<td>-10.0</td>
<td>38.9</td>
</tr>
<tr>
<td>L</td>
<td>24.7</td>
<td>6.9</td>
<td>44.8</td>
</tr>
<tr>
<td>T0</td>
<td>53.3</td>
<td>-27.9</td>
<td>55.8</td>
</tr>
<tr>
<td>LV</td>
<td>51.5</td>
<td>26.1</td>
<td>28.9</td>
</tr>
<tr>
<td>c</td>
<td>26.7</td>
<td>-12.5</td>
<td>47.0</td>
</tr>
<tr>
<td>N4</td>
<td>89.7</td>
<td>-3.05</td>
<td>45.6</td>
</tr>
</tbody>
</table>

**Application to CF:** All least squares computations were performed using the routine NL2SOL (50). In every case, a solution was obtained after at least 30 function evaluations and 24 evaluations of the Jacobian.

Starting structures for CF were built by determining positions for the sites in the following order: site N1, site N2, site N3, site e, site D, site LV, site L, site N4, site 55. There are four distance measurements between the first seven sites to define a unique approximate starting structure for these sites. Only three distance measurements connect site N4 to the first seven sites; hence, the starting position for N4 was ambiguous. The 55 site was initially connected by distance measurements to sites N3, e, and N4 only, and was therefore ambiguous (the upper and lower bounds for the distances of site 55 from other sites failed to define its position). Thus there were four possible starting structures. Least-squares refinement of these starting structures led to two distinct models, which had nearly the same residual sum of squares. After the addition of the distance measurement between sites LV and 55, as described in the test, only two distinct starting structures could be built by the method described above. Least-squares refinement of either starting structure led to the model presented in the text. A set of coordinates is given in Table 3. It should be noted that the handedness of the model cannot be determined from the distances alone; a model in which the last six sites are reflected in the plane of the N1, N2, and N3 sites is equally likely.