Cooperativity among Manganese-binding Sites in the H+-ATPase of Chloroplasts*

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† The catalytic site for ATP synthesis and hydrolysis is located on coupling factor 1 (CF1) of the H+-ATPase in chloroplast thylakoid membranes (1). Based on an M, of 400,000, the stoichiometry of CF1 subunits was suggested to be 3α, 3β, γ, δ, and ε (2). The results obtained from various experimental approaches indicated that the active sites are located either on the α or β subunits. Thus, ATPase activity was not inhibited following tryptic digestion, which removed the smaller subunits but left the α, β, and possibly a fraction of the γ subunits (3). Chemical modifiers of specific amino acid residues, which inhibited ATPase activity, were found to be either covalently bound to the β subunit or equally distributed between both the α and the β subunits. Complete inactivation of the binding site was caused by binding 2 mol of the arginyl modifiers, naphthylglyoxal (4) and phenylglyoxal (5), and the lysine modifier pyridoxal phosphate (6) to 1 mol of CF1. These modifiers were equally distributed between the α and β subunits. The same pattern of distribution was found in the binding of the ADP analogs, 3'-O-[3-(N-azido-2-nitrophenyl)amino]-propionyl-ADP (7) and 8-azo-ADP or -ATP (8), but in these cases 1 mol of the analog/mol of CF1 was sufficient to cause inactivation of activity. Binding of 1 mol/mol of CF1 of the carboxyl modifier dicyclohexylcarbodiimide (9) and of the tyrosyl reagent 7-nitro-1,2,3-benzooxadiazolyl-4-amino chloride also caused complete inhibition of the activity. However, these modifiers (3) were found to be bound only to the β subunit. These results indicate that subunit interactions are involved in the inhibition since it is likely that the enzyme has at least three active sites, one on each of the larger subunits.

MATERIALS AND METHODS

CF1 was isolated from lettuce (romaine) chloroplasts by the sucrose extraction method (14) which was modified as follows. After six washings with 10 mM PPi, CF1 was extracted in a medium containing 300 mM sucrose, 2 mM Tris/Tricine, pH 7.8, and 0.1 mM ATP. After addition of 20 mM Tris/NaOH, pH 7.1, 2 mM EDTA, 80 mM (NH4)2SO4, and 1 mM ATP, the residual membranes were precipitated by centrifugation at 31,000 × g for 15 min. CF1 was precipitated from the medium in 2 M (NH4)2SO4. The precipitate was dissolved in 40 mM Tricine/NaOH, pH 8, 2 mM EDTA, and 1 mM ATP. The ammonium sulfate was removed by a passage through a Sephadex G-50 centrifugation column equilibrated with the same medium. The enzyme was stored in 25% glycerol, 30 mM Tricine/NaOH, pH 8, 2 mM EDTA, and 1 mM ATP at -80°C. Following heat treatment (15), the rate of hydrolysis was 35 μmol × mg of protein-1 × min-1 when measured with CaATP as substrate (12). The isolated CF1 contained very little contaminating proteins as seen on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (16). Approximately less than 5% contamination by ribulose-1,5-bisphosphate carboxylase was estimated from the intensity of the Coomassie Blue staining of the small subunits of this enzyme (12 kDa) when compared to the staining of the ε subunit of CF1 (13 kDa) on the gel. When indicated, arginine modification was performed on the chloroplasts prior to the isolation of CF1 (4).

Binding of Mn to CF1 was determined by the EPR method (11). For the binding studies, CF1 was passed on a Sephadex G-50 column (1 × 50 cm) equilibrated with 40 mM HEPES/NaOH, pH 8, and then brought by ultrafiltration to the concentration of 50 μM protein. 100-μl samples were titrated by consecutive additions of 0.5 μl of concentrated MnCl2 solution. Following incubation for 15 min at 25°C, the samples were inserted into 50-μl capillary tubes for measurement of the EPR spectra in a Varian E-3 spectrometer at 25°C. Incubation of the enzyme in even low concentration of MnCl2 up to 5 h did not change the EPR signal, indicating that equilibrium of binding was reached within 15 min. The concentration of free Mn2+ ions was determined from the amplitude of the EPR absorption spectra at the X-band frequency region as compared to a standard MnCl2 solution. These measurements were possible since the complex of Mn2+ with

1 The abbreviations used are: CF1, chloroplast coupling factor 1 of H+-ATPase; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-glycine; HEPES, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid.

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CF1 was found to present an extremely broad spectrum at this frequency region while the free Mn2+ ions had a sharp characteristic six-band spectrum. Protein concentration was calculated from the absorption at 280 nm (17) assuming a M_r of 400,000 (2).

Mn2+ binding data were plotted according to the Scatchard method. Non-interacting binding sites were statistically analyzed using the maximum likelihood principle (18) with the aid of a computer program (developed by Dr. J. Hoebberg and J. Gierer, Tel Aviv University) using a CDC 8000 computer. Interacting binding sites were determined by a semi-empirical method (19).

**RESULTS**

**Interacting Mn2+-binding Sites in CF1**—The data which were previously obtained from titration of CF1, stored as (NH4)2SO4 precipitate, with MnCl2 gave descending nonlinear curves when plotted according to Scatchard analysis (12). For the analysis of these data, it was sufficient to assume that the isotherms represent binding of Mn2+ to two types of non-interacting sites. The results obtained from titration of CF1 stored in a medium containing 1 mM ATP and 25% glycerol at −80 °C gave an isotherm which curved upward to a maximum and then descended nonlinearly (Fig. 1). Such a complicated isotherm of a Scatchard plot was assumed to represent at least two types of binding sites: one type of interacting site with a positive cooperativity which gave a downward concave curve and a second type of non-interacting site which gave a descending isotherm (19). In this composite interaction, moles of Mn2+ bound per mole of enzyme (ν) and ν/C are related to the number of binding sites (N), their association constant (K), the concentration of free Mn2+ (C), and the Hill coefficient (α) as the following:

\[
\begin{align*}
\nu &= \frac{N_{1}K_{1}C^{1}}{1 + K_{1}C} + \frac{N_{2}K_{2}C^{2}}{1 + K_{2}C} \\
\frac{\nu}{C} &= \frac{N_{1}K_{1}C^{1-1}}{1 + K_{1}C} + \frac{N_{2}K_{2}}{1 + N_{2}K_{2}C^{2}}
\end{align*}
\]  

Subscripts I and S indicate interacting and non-interacting sites, respectively. The first part of each of the two equations represents the contribution to ν and ν/C of the interacting sites, while the second part represents the contribution of the non-interacting sites. In order to resolve the composite data, an empirical asymptotic tangent line to the descending part of the curve was drawn. This tangent was drawn in a manner which neglected some of the very weak non-interacting sites. From its intercept at the ν/C ordinate, the ν ordinate, and from the intercept of the original curve with the ν/C ordinate, the N_I, N_S, and K were obtained. The values of ν and ν/C of the interacting sites were calculated from equations 1 and 2 using these parameters. If the plot of ln C versus ln(N_I/ν+I) yielded a straight line (Equation 3), then from the slope and intercepts, α and K could be calculated (for details of calculations see Ref. 19).

\[
\ln C = -\frac{1}{\alpha} \ln \left( \frac{N_{I}}{\nu} - 1 \right) - \ln k_{i}
\]  

**Dependence of Mn2+ Binding on Pretreatment of CF1**—The difference in the manner of Mn2+ binding to CF1, stored as ammonium sulfate precipitate at 4 °C and to an enzyme stored in glycerol at −80 °C could not be explained by a difference in ligand content of the two enzymes, since the mode of storage did not alter the nucleotide and Mg2+ content of the enzyme. Under both conditions, CF1 contained slightly more than 1 mol of nucleotide, mostly ADP, and 1 mol of Mg2+ per mol of CF1 (Table II). It was found, however, that CF1 stored as an ammonium sulfate precipitate can show cooperative interaction among Mn2+-binding sites if after the removal of
the salt the enzyme was either preincubated overnight with 1 mM ATP or heat-activated. The number of interacting sites and their $K_d$ values were similar to the results obtained with CF, stored in glycerol (not shown).

The pretreatment of CF, resulted also in a difference in the effect of ATP on Mn$^{2+}$ binding. As we have shown earlier (12), there was no cooperativity in Mn$^{2+}$ binding to CF, stored as an ammonium sulfate precipitate. One strong and five loose binding sites with $K_d$ values of 3.8 and 188 $\mu$m, respectively, were found. Addition of 1 mol of ATP/mole of CF, caused a 200-fold decrease in the $K_d$ of one of the loose binding sites, resulting in the presence of two strong and four loose binding sites with $K_d$ values of 1 and 50 $\mu$m, respectively. However, the addition of 1 mol of either ATP or MgATP to CF, which was preincubated with ATP (the ATP was removed before the Mn$^{2+}$ binding assay) only slightly decreased the $K_d$ of Mn$^{2+}$ binding to the three cooperative sites. Thus, in the presence of MgATP, the $K_d$ decreased from 14.7 to 5.6 $\mu$m for the three interacting Mn$^{2+}$-binding sites (Table I).

Effect of Modification by Naphthylglyoxal on Mn$^{2+}$ Binding to CF,—Following the treatment of thylakoids with naphthylglyoxal, the isolated CF, had only 15% of its ATPase activity after heat activation. Unlike the nontreated CF, the naphthylglyoxal-modified enzyme bound Mn$^{2+}$ in a noncooperative manner (Fig. 4c), although both types of enzymes were similarly stored in glycerol at $-80^\circ$C. Addition of 1 mol of ATP/mole of CF, caused a great increase in the affinity of Mn$^{2+}$ to the modified enzyme (Fig. 4b). Analysis of these data indicated (Table I) that the modified enzyme had two stronger and four weaker binding sites for Mn$^{2+}$. In the presence of ATP, one very tight and five loose Mn$^{2+}$-binding sites were measured. This pattern of Mn$^{2+}$ binding to the modified enzyme differs from binding to the untreated enzyme stored in ammonium sulfate since the latter had two tight and four loose binding sites in the presence of ATP.

**DISCUSSION**

The study of substrate binding to an enzyme is an important tool for the understanding of the mode of action of catalysis. CF, has multiple binding sites for the substrate, yet substrates were found to be also effectors of the activity (1). Based on binding and exchange studies, it was suggested (7) that the two tight nucleotide-binding sites have a regulatory function, while one of the loose binding sites functions in catalysis; yet during phosphorylation, transient tight binding of nucleotides which was kinetically competent for catalysis was found (10). The apparent contradiction was partly explained by suggesting cooperative interaction among the catalytic sites. The cooperativity among the tight Mn$^{2+}$-binding sites demonstrated in this work is suggested by us to be a result of such a cooperative interaction among the catalytic sites of the enzyme. Support for this suggestion comes from the fact that modification of arginine residues, which inhibited ATPase activity, also inhibited the cooperative interaction in Mn$^{2+}$ binding. The good correlation between binding and function could be interpreted to indicate that such modification inhibited the interaction required for catalysis. The fact that a modification of only 1 arginine was sufficient to cause inhibition (4) while there are suggested to be three catalytic sites, one per each of the larger subunits of the enzyme, also indicated that subunit interaction is involved in the mode of

**TABLE I**

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Additions</th>
<th>$N_0$</th>
<th>$N_2$</th>
<th>$K_d$</th>
<th>$\alpha$</th>
<th>$K_a$</th>
<th>$\alpha$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>3</td>
<td>0</td>
<td>14.70±0.44</td>
<td>2.9±0.20</td>
<td>0</td>
<td>3</td>
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<tr>
<td>None</td>
<td>MgATP</td>
<td>3</td>
<td>0</td>
<td>5.70±0.37</td>
<td>3.07±0.30</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Naphthylglyoxal</td>
<td>None</td>
<td>0</td>
<td>2</td>
<td>33.80±3.10</td>
<td>1</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Naphthylglyoxal</td>
<td>ATP</td>
<td>0</td>
<td>1</td>
<td>3.11±0.62</td>
<td>1</td>
<td>0</td>
<td>5</td>
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</tbody>
</table>

**TABLE II**

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Mg$^{2+}$/CF$_1$</th>
<th>ADP/CF$_1$</th>
<th>ATP/CF$_1$</th>
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</thead>
<tbody>
<tr>
<td>(NH$_4$)$_2$SO$_4$, 4 °C</td>
<td>1.55±0.12</td>
<td>1.03±0.21</td>
<td>0.27±0.02</td>
</tr>
<tr>
<td>25% glycerol, $-80^\circ$C</td>
<td>1.38±0.07</td>
<td>1.28±0.25</td>
<td>0.27±0.05</td>
</tr>
</tbody>
</table>

**FIG. 3.** Scatchard plot of the calculated Mn$^{2+}$ binding to the interacting sites in CF$_1$. The data for the interacting Mn$^{2+}$ binding sites (Fig. 2) were plotted according to Scatchard.
inhibition. A similar type of interaction was observed in the action of various other modifiers (3-9). Since in the presence of ATP only one tight Mn$^{2+}$-binding site was left, it is tempting to speculate that the modification of an arginine near one of the tight Mn$^{2+}$-binding sites weakened the binding capacity to this site as well as the interaction with other sites. The suggestion that the tight binding of the Mn$^{2+}$ is at the active sites of the enzyme is also supported by our previous work. In CF$_1$ stored as an ammonium sulfate precipitate, we identified in the presence of ATP only two tight binding sites with a $K_d$ slightly lower than those measured for the three sites in this work. It is possible that after the removal of the salt, CF$_1$ was in a conformation which prevented cooperative interaction and therefore the third site could not tightly bind Mn$^{2+}$. Yet the similarity in the effect of the effectors and in the changes of the pH of the medium on the kinetic and on the binding parameters suggested that the two tight binding sites could be at the active site (11-13).

The transition from non-interacting to cooperative binding mode of Mn$^{2+}$ seems to be related to interaction of CF$_1$ with ATP at a low affinity site. This suggestion is based on the finding that preincubation of CF$_1$ which was stored as an ammonium sulfate precipitate either with 1 mM ATP overnight or in 1 mM ATP and 25% glycerol at -80 °C or heat activation in the presence of 40 mM ATP yielded an enzyme which bound Mn$^{2+}$ in a cooperative manner. Based on these results, it is also suggested that the active enzyme has the capacity for cooperative interaction. Cooperative interaction among ATP-binding sites was demonstrated in F$_1$ from bacteria and mitochondria (21, 22).

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REFERENCES