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Assessment of the Number of Nucleotide Binding Sites on Chloroplast Coupling Factor 1 by the Continuous Variation Method†

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ABSTRACT: The method of continuous variation (Job plot analysis) and difference absorbance spectroscopy were used to investigate the binding of 2′(3′)-(trinitrophenyl)-ADP and -ATP to chloroplast coupling factor 1 (CF_1). Experiments performed at a low total concentration (30 μM) of nucleotide and enzyme binding sites (assuming three or four binding sites per CF_1) could be interpreted in terms of approximately three nucleotide binding sites per CF_1. At higher total concentrations (100 and 400 μM), the number of apparent binding sites increased to almost four. Computer-generated Job plots, using a protein–ligand complex formation scheme of n independent, nonequivalent binding sites, gave good fits to the experimental data at all concentrations when four binding sites were modeled. The dissociation constant of the fourth site was estimated to be $\sim$20 μM. Additional nucleotide binding sites were not directly observed by this method and, if they exist, have very weak binding affinities (dissociation constants $>\sim$1 mM).

The soluble, extrinsic portion of the ATP synthetase isolated from spinach chloroplasts, chloroplast coupling factor 1 (CF_1),[1] is comprised of five types of polypeptides (α, β, γ, δ, and ϵ). The two largest polypeptides, α and β, have been associated with nucleotide binding (Kambouris & Hammes, 1985; Admon & Hammes, 1987). The stoichiometry of the polypeptide chains comprising CF_1, $\alpha_3\beta_3\gamma\delta\epsilon$ (Moroney et al., 1983), is identical with that of F_1 sectors isolated from proton ATPases of bacteria and mitochondria [cf. Senior and Wise (1983)]. Recent studies with *Escherichia coli* and mitochondrial F_1 provide strong evidence for the existence of six distinct nucleotide binding sites (Wise et al., 1983; Kironde & Cross, 1986). Ligand binding studies with rat liver mitochondrial

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[1] Abbreviations: CF_1 and ECF_1, F_1-ATPases isolated from spinach chloroplasts and *Escherichia coli*, respectively; EDTA, ethylenediaminetetraacetic acid; TNP-AMP, -ADP, or -ATP, 2′(3′)-(trinitrophenyl)adenosine mono-, di-, or triphosphate, respectively; Tris, tris(hydroxymethyl)aminomethane.

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F_1, on the other hand, have detected only four nucleotide binding sites (Williams et al., 1987). Three nucleotide binding sites on CF_1 have been well characterized: two catalytic sites for ATP synthesis and hydrolysis and a noncatalytic site (Bruist & Hammes, 1982; Leckband & Hammes, 1987). While equilibrium binding measurements have detected only three binding sites (Bruist & Hammes, 1981), photolabeling studies with 2-azido nucleotides and Sephadex chromatography experiments have been interpreted in terms of more than three binding sites (Xue et al., 1987). Unfortunately, these experiments do not distinguish between specific and nonspecific binding. The present study was undertaken to investigate further the nucleotide binding characteristics of CF_1 using the method of continuous variation (Job, 1928).

The continuous variation method, or Job plot analysis, was originally developed to determine the composition of metal–ligand complexes in solution. This method, which involves measurement of complex formation at various combinations of mole fractions of the reactants while maintaining a constant total concentration of reactants, also has been applied to protein–ligand interactions [cf. Hammes et al. (1970) and Prakash and Timasheff (1983)]. The Job method depends on the existence of a measurable parameter that is proportional to complex formation. The existence of a difference absorbance spectrum upon binding of TNP-ADP and TNP-ATP to CF_1, and the relatively tight binding of these nucleotide analogues to coupling factors, makes them good probes for this investigation. Moreover, the presence of isosbestic points in the difference spectra was used to establish the specificity of binding.

Materials and Methods

Materials. The sources of the chemicals were as follows: ADP and ATP (vanadium free) were from Sigma Chemical Co.; TNP-AMP, TNP-ADP, and TNP-ATP were from Molecular Probes. Silica gel thin-layer chromatography plates with fluorescent indicator were from Machery-Nagel. All other chemicals were high-quality commercial grades, and all solutions were prepared from deionized water. TNP-ATP was purified by column chromatography on Bio-Rad AG-1-X4 Cl^- (1 × 10 cm). The column was washed with 500 mM NaCl–20 mM HCl, and the TNP-ATP was eluted with 5 M NaCl–20 mM HCl. The eluent was neutralized with 7 M NH_4OH, and the TNP-ATP was precipitated with 0.2 mL of 2 M barium acetate/mL of solution. Following a 12-h incubation at 4 °C, the precipitate was washed with water and solubilized by addition of Dowex 50W-X8 H^+ resin (Sigma). The TNP-ATP was stored at pH 7–9. The TNP-ATP, -ADP, and -AMP were judged to be >95% pure by thin-layer chromatrography on silica gel plates using a solvent system of dioxane–2-propanol–11 N NH_4OH–H_2O, 40:20:36:34 v/v (Boulay et al., 1985). The R_f values for TNP-AMP, TNP-ADP, and TNP-ATP were 0.84, 0.61, and 0.53, respectively.

Enzyme. CF_1 was prepared from fresh market spinach as previously described (Lien & Racker, 1971; Binder et al., 1978). The purified enzyme had a fluorescence emission ratio, 305/340 nm (excitation 280 nm), greater than 1.5. The enzyme was precipitated with an equal volume of saturated ammonium sulfate in 10 mM Tris-HCl (pH 7.2) and 1 mM EDTA and stored at 4 °C. Prior to use, CF_1 was desalted by passing small volumes of enzyme through two consecutive centrifuge columns (Penefsky, 1977).

The ECF_1 used in these experiments was a gift from A. L. Senior (University of Rochester). Before use, ECF_1 was depleted of nucleotides by passage through a column (1 × 60 cm) of Sephadex G-25 equilibrated with 50% (v/v) glycerol, 100 mM Tris–H_2SO_4, and 4 mM EDTA, pH 8.0, and eluted at 3.0 mL/h at room temperature (Wise et al., 1983). Peak fractions ($A_{280}/A_{260} > 1.70$) were pooled and precipitated with 67% saturated ammonium sulfate. Prior to use, the enzyme was dissolved in 50 mM Tris–H_2SO_4 and 0.5 mM EDTA, pH 8.0, and desalted on a centrifuge column in the same buffer.

Difference Spectrophotometry. Difference spectra were recorded on a Cary 118 spectrophotometer at 25 °C. Tandem cells with a path length of 0.45 cm/cell compartment were employed for most measurements. The difference spectrum measured was the absorbance of the enzyme–ligand mixture minus that of the same concentrations of enzyme and ligand. For difference spectra recorded at extremely high concentrations of protein and ligand, cells with a path length of 0.10 cm were used. Spectra were recorded from 350 to 600 nm.

Method of Continuous Variation. The method of continuous variation (Job, 1928; Asmus, 1961) was employed to investigate binding of nucleotide analogues to CF_1. The total concentration of protein sites and ligand was held constant, while their relative concentrations were varied. The concentration of protein sites was calculated by assuming a specific binding stoichiometry for each experiment. Complex formation was monitored by measuring the difference absorbance, ΔA.

For each binding experiment with CF_1, the total concentrations employed ranged from 30 to 400 μM, assuming either three or four nucleotide binding sites per CF_1 molecule. Concentrations of CF_1 were determined by use of an extinction coefficient of 0.483 mL/(mg·cm) at 277 nm (Bruist & Hammes, 1981) and a molecular weight of 400 000 (Moroney et al., 1983). When necessary, protein was concentrated with Centricon-10 microconcentrators from Amicon. The concentrations of the TNP nucleotides were determined by assuming an extinction coefficient of 26 400 M^{-1} cm^{-1} at 408 nm, pH 8.0 (Hiratsuka & Uchida, 1973). All stock solutions were prepared in 50 mM Tris-HCl and 1 mM EDTA, pH 8.0. In most experiments, 5 mM Mg^{2+} was also present. Clean glass pipets were siliconized with Sigmacote (Sigma) before use. Incubations with TNP nucleotides were carried out at room temperature for 12–18 h to ensure that binding of the ligand was complete.

ATP and ADP chase experiments were performed by first incubating CF_1 with the TNP nucleotide for 12 h at the mole ratio corresponding to maximum binding. A 400-fold excess of ADP or ATP was then added from 250 mM stock solutions. Difference spectra of a control sample and the chased samples were recorded following 3- and 20-h incubation periods. The concentrations of ATP and ADP were determined by using an extinction coefficient of 15 400 M^{-1} cm^{-1} at 259 nm (Beaven et al., 1955).

An experiment with ECF_1 was performed at 30 μM total concentration of ECF_1 sites plus TNP-ADP. A molecular weight of 382 000 and six binding sites per ECF_1 molecule were assumed in this calculation (Senior & Wise, 1983). Protein concentration was determined by using a modification of the Lowry method (Bensadoun, 1976). Sample preparation and incubations with nucleotide were performed as for CF_1 in 50 mM Tris–H_2SO_4, 0.5 mM EDTA, and 5 mM Mg^{2+}, pH 8.0.

Theory

The theoretical basis for the method of continuous variation has been described for various schemes of protein–ligand complex formation, including that of n equivalent, independent binding sites (Huang, 1982). If the sum of the protein and ligand concentrations is held constant while the mole fraction of the ligand is varied, the measured physical parameter, ab-

Table I: Difference Spectra Characteristics

nucleotide	metal	peaks and troughs (nm)	isosbestic points (nm)	ΔA_{max}[a] (nm)
TNP-ATP	Mg^{2+}	505, 450, 419, 390	475, 423, 413	419–390
TNP-ADP	Mg^{2+}	505, 450, 419, 390	475, 428, 414	419–390
	Ca^{2+}	507, 454, 419, 390	485, 428, 414	419–390
	none	510, 460, 422, 390	490, 435, 414	422–390
TNP-AMP	Mg^{2+}	420, 390	poorly defined	420–390
TNP-ADP[b]	Mg^{2+}	510, 450, 419, 390	490, 425, 410	510–450

[a] Wavelengths in the difference spectrum giving the largest difference absorbance between an adjacent peak and trough. [b] ECF_1 rather than CF_1.

sorbance in this case, goes through a maximum at a mole fraction determined by the binding stoichiometry. This method is reliable if the sum of the ligand and protein concentrations is much greater than the dissociation constant for the ligand–protein interaction. This concept can be readily extended to the case of n independent, nonequivalent binding sites. The absorbance, A, of a protein–ligand mixture is

$$A = l(\epsilon_E[E] + \epsilon_L[L] + \sum_{i=1}^{n}\epsilon_i[EL]_i) \quad (1)$$

where [E], [L], and $[EL]_i$ represent free enzyme, free ligand, and the enzyme-ligand complex on site i, respectively, l is the path length, and the ϵ_i values are extinction coefficients. The difference absorbance, ΔA, is

$$\Delta A = l\sum_{i=1}^{n}(\epsilon_i - \epsilon_L - \epsilon_E)[EL]_i \quad (2)$$

If the difference extinction coefficient is the same for each ligand bound to the enzyme

$$\Delta A = l\Delta\epsilon\sum_{i=1}^{n}[EL]_i \quad (3)$$

Since ΔA is proportional to the sum, which is the total concentration of bound ligand, $[L_b]$, it is apparent that under the conditions used for the method of continuous variation ΔA and $[L_b]$ have the same dependence on the concentration. For n independent, nonequivalent binding sites and a total enzyme concentration of $[E_0]$

$$[L_b] = \sum_{i=1}^{n}\frac{[L]/K_i}{1 + [L]/K_i}[E_0] \quad (4)$$

For a continuous variation experiment, the total concentration of enzyme sites and ligand, $[C_0]$, is

$$[C_0] = s[E_0] + [L_0] \quad (5)$$

where s is the assumed number of binding sites and $[L_0]$ is the total ligand concentration. Since $[L_0] = [L_b] + [L]$

$$[L_b] = \frac{\sum([C_0] - [L])}{s + \sum} \quad (6)$$

where the sum in eq 4 is represented by $\sum$. The mole fraction of ligand, X_L, is

$$X_L = ([L_b] + [L])/[C_0] \quad (7)$$

Computer plots of $[L_b]$ (or equivalently ΔA for known l and $\Delta\epsilon$) versus X_L can be readily generated from eq 6 and 7 for given values of $[C_0]$, s, [L], and the K_i. Examples of these theoretical Job plots will be given later.

To determine when $[L_b]$ (or ΔA) goes through a maximum, the free ligand concentration, [L], in eq 4 can be expressed in terms of X_L, $[C_0]$, and $[L_b]$ by use of eq 7. The resulting equation can be solved for $[L_b]$, and if $d[L_b]/dX_L$ is set equal to 0, the mole fraction at which $[L_b]$ goes through a maximum can be calculated. For example, if $s = 1$ and two binding sites are present, the maximum occurs at $X_L = {}^2/_3$ if $[C_0] \gg K_1$, K_2. This is the result expected for two binding sites. Although the general case has not been proved analytically, a sufficient number of cases have been examined analytically and through computer plots to suggest that the maximum provides a reliable stoichiometry if $[C_0] \gg K_1, K_2, K_3, \ldots, K_n$.

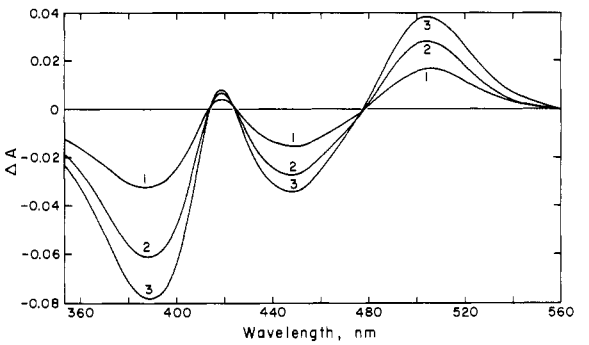

FIGURE 1: Absorbance difference spectra of TNP-ATP bound to CF_1. Spectra were recorded on a Cary 118 spectrophotometer with tandem cells of path length 0.45 cm/cell compartment. The sample cell contained CF_1 and TNP-ATP in compartment a and buffer in compartment b. The reference cell contained CF_1 alone in compartment a and TNP-ATP in compartment b. For each spectrum, the total concentration of CF_1 binding sites (calculated by assuming three binding sites per CF_1) and TNP-ATP was 100 μM, while the mole fraction of TNP-ATP was 0.15 (spectrum 1), 0.30 (spectrum 2), and 0.40 (spectrum 3). The buffer was 50 mM Tris-HCl, 1 mM EDTA, and 5 mM Mg^{2+}, pH 8.0.

RESULTS

The binding of TNP-ATP to CF_1 in the presence of Mg^{2+} produces difference spectra such as those in Figure 1. In these samples, the total concentration of enzyme sites and ligand was held constant at 100 μM. The spectra shown are at three different mole ratios of CF_1 and TNP-ATP and are characterized by peaks at 505 and 419 nm and troughs at 450 and 390 nm. Well-defined isosbestic points at 475, 423, and 413 nm also are present. Similar difference spectra were obtained upon binding of TNP-ADP to CF_1 in the presence of Mg^{2+}. Table I summarizes features of the various difference spectra. To investigate the effect of metal on the binding of TNP-ADP to CF_1, experiments were performed in the presence of Mg^{2+} and Ca^{2+} and in the absence of metal. A small metal-dependent shift in the difference spectrum is observed. All spectra recorded with TNP-ATP and TNP-ADP bound to CF_1 or ECF_1 displayed well-defined peaks and isosbestic points, suggestive of specific binding. The peaks in the CF_1–TNP-AMP difference spectrum, on the other hand, were much broader. Additionally, these spectra did not possess clearly defined isosbestic points, suggesting weak and nonspecific binding.

Figure 2 shows the difference absorbance as a function of the mole fraction of nucleotide at three different total concentrations of CF_1 binding sites and TNP-ATP. Experimental data are indicated by circles, and the solid curves correspond to computer-generated plots, which will be discussed later. The extrapolated linear portions of the experimental data (dashed

Table II: Summary of Job Plot Characteristics

nucleotide	metal[a]	$[C_0]^b$ (μM)	$\Delta A_{max}{}^c$	$X_{L_{max}}{}^d$	n^e	$\Delta\epsilon$/site (cm^{-1} M^{-1})
TNP-ATP	Mg^{2+}	30	0.037	0.50	3.0	5500
	Mg^{2+}	100	0.126	0.55	3.7	5100
	Mg^{2+}	400	0.492	0.55	3.7	5000
TNP-ADP	Mg^{2+}	30	0.041	0.50	3.0	6000
	Mg^{2+}	100	0.151	0.55	3.7	6000
	Ca^{2+}	30	0.049	0.53	3.4	6800
	none	30	0.051	0.40	2.0	9400
TNP-ADP[f]	Mg^{2+}	30	0.030	0.45	4.9	4900

[a] 5 mM. [b] Total concentration of enzyme binding sites and nucleotides, assuming three binding sites per CF_1 and six binding sites per ECF_1 (last entry in table). [c] Calculated from the intercept of extrapolated lines; 0.45-cm path length. [d] Mole fraction of ligand corresponding to maximum in Job plot. [e] Number of binding sites obtained from $X_{L_{max}}$. [f] ECF_1 rather than CF_1.

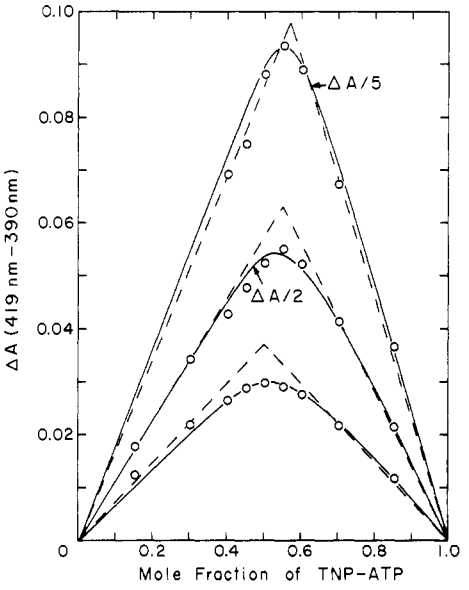

FIGURE 2: Binding of TNP-ATP to CF_1. Difference absorbance (0.45-cm cell path length) vs mole fraction of ligand. The total constant sums of concentrations of TNP-ATP and enzyme binding sites (assuming three binding sites per CF_1) were 30 μM (lower curve), 100 μM (middle curve), and 400 μM (upper curve). Experimental data are indicated by circles. Dashed lines are extrapolated linear portions of the experimental curve. Solid lines are computer-generated plots modeling four binding sites with $\Delta\epsilon$ = 5100 cm^{-1} M^{-1} and dissociation constants of $K_1 = K_2 = 0.1$ μM, $K_3 = 1.0$ μM, and $K_4 = 20.0$ μM.

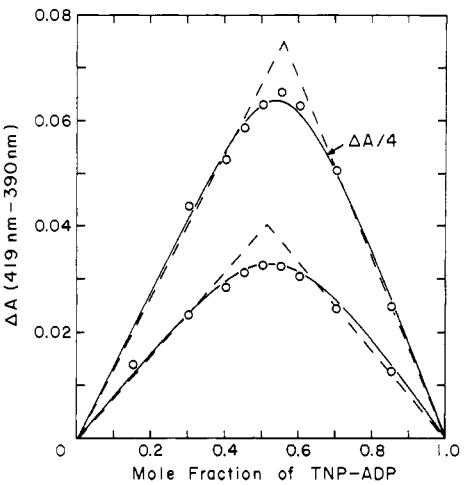

FIGURE 3: Binding of TNP-ADP to CF_1. Difference absorbance (0.45-cm cell path length) vs mole fraction of ligand. The total constant sums of concentrations of TNP-ADP and enzyme binding sites (assuming three binding sites per CF_1) were 30 μM (lower curve) and 100 μM (upper curve). Experimental data are indicated by circles. Dashed lines are extrapolated linear portions of the experimental curve. Solid lines are computer-generated plots modeling four binding sites with $\Delta\epsilon$ = 6350 cm^{-1} M^{-1} and dissociation constants of $K_1 = 0.6$ μM, $K_2 = 1.0$ μM, $K_3 = 3.0$ μM, and $K_4 = 20.0$ μM.

lines) were generated by fixing the points at $X_L = 0$ and $X_L = 1$ and fitting the next two or three data points to a straight line. In these experiments, binding was performed in the presence of Mg^{2+}, and the concentration of CF_1 sites was calculated by assuming three sites per CF_1. To decrease the effects of instrument noise and shifts in starting positions of individual spectra, difference absorbances were calculated as the difference between the peak at 419 nm and the trough at 390 nm in the difference spectrum. At 30 μM, a maximum difference absorbance was observed at a mole fraction of 0.50, which corresponds to three nucleotide binding sites. The extrapolated lines also intersect at the same ratio. As expected, when bound TNP-ATP was chased with a 400-fold excess of ATP or ADP, the observed difference absorbance decreased by 64% over a 20-h period. The remaining TNP-ATP is probably bound at the site which binds MgATP very tightly (Bruist & Hammes, 1981). At a total concentration of 100 μM, the mole fraction corresponding to the maximum difference absorbance, 0.55, again agrees with the mole fraction at which the extrapolated lines intersect, and corresponds to 3.7 nucleotide binding sites. At 400 μM total concentration, a maximum difference absorbance also occurs at a mole fraction of 0.55. The extrapolated linear portions of the data, however, intersect at a mole fraction of 0.57, corresponding to four binding sites. These results are summarized in Table II. Also listed in this table are values of $\Delta\epsilon$, the difference extinction coefficient. Values of $\Delta\epsilon$ per site were calculated from

$$\Delta\epsilon = \Delta A_{max}/lcn$$

where ΔA_{max} is the difference absorbance ($\Delta A_{419} - \Delta A_{390}$) at the intersection of the extrapolated linear portions of the experimental curve, l is the cell path length, c is the concentration of CF_1 at the Job plot maximum, and n is the number of binding sites as determined from the mole fraction of ligand at the maximum of the curve. These values of $\Delta\epsilon$ are consistent for all experiments performed with TNP-ATP (standard deviation ±7%). An additional experiment was performed to determine $\Delta\epsilon$ per site for TNP-ATP binding to the sites that bind ADP and MgATP very tightly (Bruist & Hammes, 1981). This was accomplished by incubating CF_1 overnight with stoichiometric quantities of TNP-ATP and 5 mM Mg^{2+}. The difference extinction coefficient for the two sites was calculated as above, and the results were consistent with those shown in Table II: $\Delta\epsilon$ was found to be 9400 cm^{-1} M^{-1}, i.e., 4700 cm^{-1} M^{-1}/site.

Results of TNP-ADP binding experiments using the method of continuous variation also are summarized in Table II. Plots for studies with CF_1 in the presence of Mg^{2+} are shown in Figure 3. Once again, for both 30 and 100 μM total concentrations, the maxima in the experimental data and the

intersection of the extrapolated linear portions of the curve correspond very closely. Both occur at a mole fraction of 0.50 and 0.55 for 30 and 100 μM, respectively. Furthermore, when bound TNP-ADP was chased with a 400-fold excess of ATP or ADP, almost complete disappearance of the difference spectrum was observed (88%) in 20 h. Identical values of $\Delta\epsilon$ per site were calculated at 30 and 100 μM, and these are slightly larger than those obtained for TNP-ATP binding.

TNP-ADP binding experiments at 30 μM also were performed in the presence of Ca^{2+}. The maximum difference absorbance occurred at a mole fraction of 0.53 (3.4 sites), while the intersection of the extrapolated lines occurred at a mole fraction of 0.54 (3.5 sites). In an experiment performed in the absence of any metal, however, the difference absorbance reached a maximum at a much lower mole fraction, 0.40, corresponding to only two binding sites. The intersection of the limiting slopes again occurred at a slightly larger mole fraction, 0.43, or 2.3 binding sites. As shown in Tables I and II, both the appearance of the difference spectrum and the values of $\Delta\epsilon$ per site are metal dependent.

In order to be certain that the initial assumption of three binding sites was not influencing the experimental results, several experiments were carried out in which four binding sites per CF_1 were assumed in fixing the total concentration of binding sites and ligand. The binding stoichiometries obtained from the Job plots in these cases were not significantly different from those found with the assumption of three binding sites per CF_1.

As previously mentioned, the appearance of difference spectra recorded after binding TNP-AMP to CF_1 indicated relatively weak and possibly nonspecific binding. The maximum difference absorbance observed with a total concentration of binding sites and ligand of 30 μM was 0.017, which is about half as large as that observed when TNP-ADP or TNP-ATP binds to CF_1 under similar conditions. Therefore, the method of continuous variation could not be used to determine a reliable binding stoichiometry. When bound TNP-AMP was chased with excess ATP or ADP for 20 h, a 92% reduction of the difference absorbance occurred.

The appearance of the ECF_1–TNP-ADP difference spectrum in the presence of Mg^{2+} is similar to that of CF_1. As shown in Table I, however, the maximum difference absorbance occurs between the peak at 510 nm and the trough at 450 nm in the difference spectrum. The data in Figure 4 were obtained by using the method of continuous variation and a total concentration of 30 μM (assuming six binding sites per ECF_1). The maximum experimental difference absorbance occurs at a mole fraction of 0.45, corresponding to 4.9 binding sites. The extrapolated linear portions of the data, however, intersect at a ratio of 0.50, suggesting a higher binding stoichiometry of 6:1.

Computer plots of difference absorbance versus mole fraction of nucleotide were generated as described earlier. TNP nucleotide binding was modeled at 30, 100, and 400 μM total concentrations for a wide range of values of dissociation constants. Figure 2 shows the computer plots (solid lines) generated for TNP-ATP binding to CF_1, assuming three binding sites in calculating $[C_0]$ ($s = 3$) and four actual binding sites ($n = 4$). At all concentrations, plots were generated by using a value for $\Delta\epsilon$ per site of 5100 cm^{-1} M^{-1}. The values of the dissociation constants which gave a good fit to all three sets of experimental data were $K_1 = K_2 = 0.1$ μM, $K_3 = 1.0$ μM, and $K_4 = 20.0$ μM. These are not, however, unique parameters; other sets of constants also fit the data. For example, the data at 30 μM could be fit quite well to a three

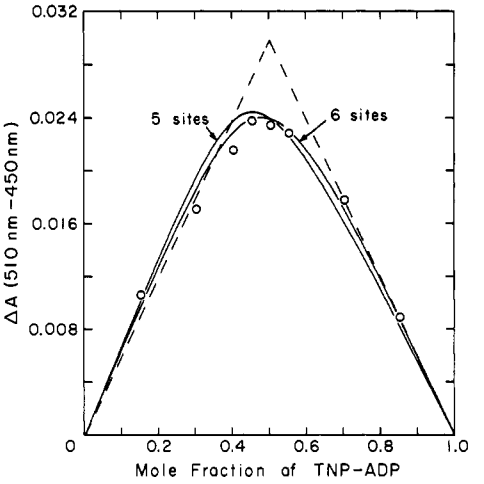

FIGURE 4: Binding of TNP-ADP to ECF_1. Difference absorbance (0.45-cm cell path length) vs mole fraction of ligand. The total constant sum of concentrations of TNP-ADP and enzyme binding sites (assuming six binding sites per ECF_1) was 30 μM. Experimental data are indicated by circles. Dashed lines are extrapolated linear portions of the experimental curve. Solid lines are computer-generated plots modeling five or six binding sites with $\Delta\epsilon = 5100$ cm^{-1} M^{-1} (five sites) and 4850 cm^{-1} M^{-1} (six sites). Dissociation constants for both models were $K_1 = K_2 = 0.1$ μM, $K_3 = K_4 = 1.0$ μM, $K_5 = 3.0$ μM, and $K_6 = 10.0$ μM.

binding site model, assuming $\Delta\epsilon$ per site = 5700 cm^{-1} M^{-1} and dissociation constants of 0.1, 1.0, and 3.0 μM. The data at the two higher concentrations, however, gave poor fits to three binding site models and good fits to four binding site models. Fits to five and six binding site models also were attempted. At 100 μM total concentration, these fits were poor unless K_5 and K_6 were >500 μM. At 400 μM total concentration, the lower limits for K_5 and K_6 were about 1 mM.

Computer-generated plots also gave good fits for TNP-ADP binding data when four binding sites were modeled (solid lines in Figure 3). Values for the dissociation constants for sites 1, 2, and 3 were raised slightly over those used for the TNP-ATP model. At both 30 and 100 μM, the following values were used: $\Delta\epsilon$ per site = 6350 cm^{-1} M^{-1}, $K_1 = 0.6$ μM, $K_2 = 1.0$ μM, $K_3 = 3.0$ μM, and $K_4 = 20.0$ μM.

The data for ECF_1 presented in Figure 4 were modeled for four, five and six binding sites using values of 0.1, 0.1, 1.0, 1.0, 3.0, and 10.0 μM for the six dissociation constants. The results for the five and six binding site models are shown in Figure 4 (solid lines). The four binding site model did not give a satisfactory fit of the data.

Discussion

As a means of testing the method of continuous variation, or Job plot analysis, ECF_1, known to contain six nucleotide binding sites, was employed. TNP-ADP binds tightly to ECF_1 in the presence of Mg^{2+}, and furthermore, binding results in a measurable difference absorbance. At a total concentration of enzyme sites and TNP-ADP of 30 μM (assuming six binding sites per ECF_1), the maximum in the Job plot occurred at a mole ratio which corresponded to a total of 4.9 binding sites. Because this method is limited to detecting binding sites with a ligand dissociation constant less than the total constant concentration employed in the experiment, this was the expected result [cf. Senior and Wise (1983)].

The difference spectra observed when TNP-ATP and TNP-ADP bind to CF_1 displayed well-defined peaks and isosbestic points which were metal dependent. In contrast, the difference absorbance spectrum observed when TNP-AMP binds to CF_1 did not contain clear isosbestic points or strong

absorbance peaks, and, therefore, this nucleotide analogue was not employed in continuous variation binding experiments. The data are consistent with the binding at each site being characterized by the same value of $\Delta\epsilon$, which suggests specific binding and similar environments at all binding sites. The fact that the difference spectra have properties that can be interpreted in terms of specific binding (in contrast to nonspecific binding) is essential for these experiments.

The results obtained with TNP-ATP and TNP-ADP have many common characteristics. At low concentration (30 μM binding sites and ligand, assuming three binding sites per CF_1), in the presence of Mg^{2+} or Ca^{2+}, 3.0–3.4 binding sites were detected. Experiments performed at higher total constant concentrations, 100 and 400 μM, resulted in consistently higher binding stoichiometries. The data with MgTNP-ATP show the clearest trend, with the number of apparent binding sites increasing as the total concentration of sites and ligand increases. Close to four nucleotide binding sites were detected whether three or four binding sites were assumed at the start of the experiment. In addition to these results obtained from the experimental Job plot maxima, evidence pointing toward the existence of a fourth site came from computer-generated Job plots. Computer plots were constructed to determine if a good fit of the experimental data to three, four, five, or six binding site models could be obtained. While theoretical plots were consistent with many different binding site models, depending on what values were chosen for the parameters ($\Delta\epsilon$ and dissociation constants), a four binding site model (with dissociation constants K_1–$K_3 \leq 3.0$ μM and $K_4 = 10$–30 μM) provided good fits to all experimental data. Moreover, five and six binding site models could be fit to data at the higher concentrations only if the dissociation constants of these sites were quite large (>0.5–1.0 mM).

In the absence of metal, only two sites were observed. Furthermore, a site that binds MgTNP-ATP tightly was detected by observing the decrease in the difference absorbance after an ADP or ATP chase. Sites that bind TNP-ADP or TNP-AMP tightly were not observed. These results are what would be expected for ATP, ADP, and AMP (Bruist & Hammes, 1981). As isolated, CF_1 contains one tightly bound nucleotide, ADP/CF_1 (mol/mol) (Carlier & Hammes, 1979). However, this ADP is displaced by TNP nucleotides, and therefore, this ADP binding site is detected in the continuous variation experiments (Cerione & Hammes, 1982).

These studies do not fully resolve the controversy with regard to the number of nucleotide binding sites on CF_1. When analogues are used, the possibility that the actual ligands behave differently always must be kept in mind. Nevertheless, the data suggest at least four *specific* TNP nucleotide binding sites on CF_1, and, by analogy, probably for ATP and ADP. However, even for these analogues, additional binding sites, if they exist, are characterized by extremely high dissociation constants. This study also illustrates that Job plots are useful tools for examining ligand binding. Although computer modeling of the curves is insufficient to establish the precise values of the dissociation constants, the shape of the curve is quite sensitive to the number of binding sites and the magnitudes of the dissociation constants. Modeling the shape of the curve provides supplementary information to the location of the maximum of the plot. Furthermore, although only one particular type of binding scheme has been considered (n independent, nonequivalent binding sites), other binding schemes can be treated in a similar manner.

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