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# Evaluation by Steady-State Enzyme Kinetics of the Role of Tightly Bound Nucleotides During Photophosphorylation

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## Abstract

The ATP synthetase of chloroplast membranes binds ADP and ATP with high affinity, and the binding becomes quasi-irreversible under certain conditions. One explanation of the function of these nucleotides is that they are transiently tightly bound during ATP synthesis as part of the catalytic process, and that the release of tightly bound ATP from one catalytic site is promoted when ADP and $P_i$ bind to a second catalytic site on the enzyme. Alternatively, it is possible that the tightly bound nucleotides are not catalytic, but instead have some regulatory function. We developed steady-state rate equations for both these models for photophosphorylation and tested them with experiments where two alternative substrates, ADP and GDP, were phosphorylated simultaneously. It was impossible to fit the results to the equations that assumed a catalytic role for tightly bound nucleotides, whether we assumed that both ADP and GDP, or only ADP, are phosphorylated by a mechanism involving substrate-induced release of product from another catalytic site. On the other hand, the equations derived from the regulatory-site model that we tested were able to fit all the results relatively well and in an internally consistent manner. We therefore conclude that the tightly bound nucleotides most likely do not derive from catalytic intermediates of ATP synthesis, but that substrate (and possibly also product) probably bind both to catalytic sites and to noncatalytic sites. The latter may modulate the transition of the ATP-synthesizing enzyme complex between its active and inactive states.

**Key Words:** Alternating-site catalysis; regulatory sites; substrate binding to noncatalytic sites.

## Introduction

Extensive work during the past decade has established that resting chloroplasts contain tightly bound nucleotides associated with $CF_1$,[3] the ATP-

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[3]Abbreviations used: $CF_1$, chloroplast coupling factor 1, the ATP synthetase/hydrolase of photophosphorylation; Tricine, *N*-tris(hydroxymethyl)methylglycine; NTP, NDP, nucleoside tri- or diphosphate.



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synthesizing enzyme. The tight-binding sites are then able to release and rebind ADP when the chloroplast membranes are energized by illumination, an acid–base transition, or an external electric field. The exchange of bound for free nucleotides is inhibited by uncouplers and by some energy-transfer inhibitors (see Shavit, 1980, for a review of the literature). Thus it seems that tight-binding sites for nucleotides must have some role in ATP synthesis.

Attempts have been made to understand whether tightly bound nucleotides play a catalytic or a regulatory role in the process of photophosphorylation. One model that proposes a catalytic function is the "alternating-site" or "binding-change" mechanism (Boyer and Kohlbrenner, 1981; Smith and Boyer, 1976). In this model, the ligands are transiently tightly bound during the phosphorylation step. The subsequent release of tightly bound ATP via an energy-requiring conversion of its catalytic site from a tight-binding to a loose-binding conformation occurs concomitant with conversion from loose to tight of the binding of substrates at another catalytic site on the same enzyme molecule. Since the three events (tight-to-loose ATP, loose-to-tight ADP, and enzyme conformational change) occur simultaneously and are evidently interdependent, this model is formally indistinguishable from the flip-flop mechanism analyzed for alkaline phosphatase (Bale *et al.*, 1980).

Objections to catalytic-site models have mainly centered on the question of whether the tightly bound nucleotides can exchange rapidly enough under energized conditions to be consistent with their proposed catalytic function. An alternative explanation is that the function of the tight-binding sites is not catalytic, but regulatory. Tightly bound ADP may play an important role in the active–inactive state transitions of $CF_1$, being released when $CF_1$ is activated (Bar-Zvi and Shavit, 1980; Dunham and Selman, 1981; Schumann and Strotmann, 1981; Shavit *et al.*, 1981; Strotmann *et al.*, 1981a).

In most previous studies, the tightly bound nucleotides have been analyzed directly. However, a direct analysis requires separation of the chloroplasts from the phosphorylation reaction medium, with inevitable delays and changes in the conditions. This may result in a change in the relative amounts of bound ADP and ATP (Shavit, 1980), or in loosely bound nucleotides becoming tightly bound (Strotmann *et al.*, 1981b). Tightly bound ATP has also been measured under photophosphorylation conditions by its inaccessibility to hexokinase (Shavit *et al.*, 1977; Rosen *et al.*, 1979), but recent results show that some of the ATP that is inaccessible to hexokinase nonetheless can be replaced by added excess ATP, suggesting that it may not be tightly bound (Aflalo and Shavit, 1982).

This paper represents a significantly different approach to assessing the role of bound nucleotides, because the experiments were performed exclusively under steady-state photophosphorylation conditions. Steady-state velocity equations were derived for the two models for ATP synthesis. Following

the example of Bale *et al.* (1980), experiments where ADP and GDP were phosphorylated simultaneously by the chloroplasts were used to distinguish between the models.

## Materials and Methods

### *Preparation and Assay of Chloroplast Membranes*

Chloroplast membranes were isolated from Romaine lettuce by conventional procedures and washed with 0.4 M sucrose, 1 mM tricine, pH 8.0 (Aflalo and Shavit, 1982). Photophosphorylation assay mixtures contained in a volume of 1 ml the following components ($\mu$mol): ADP, 0–0.4; GDP, 0–1.5; $MgCl_2$, 10; glucose, 20; Tricine (pH 8.0), 50; KCl, 50; phenazine methosulfate, 0.04; $^{32}P_i$, 2, containing about $1.5 \times 10^7$ cpm; as well as chloroplasts containing about 50 $\mu$g of chlorophyll; and hexokinase, 5 units. The mixtures were illuminated with saturating white light for 5 sec in a water bath maintained at 20°C, and then were allowed to incubate for about 10 sec longer in darkness to ensure that the hexokinase had completely converted the [$\gamma$-$^{32}$P]ATP to glucose-6-[$^{32}$P]phosphate. Trichloroacetic acid (0.1 ml of 30%, w/v) was added to quench the reactions. After centrifugation, two samples were taken from each quenched reaction mixture. One sample was made 1 M in HCl and boiled for 10 min to hydrolyze GTP (but not glucose-6-phosphate). Then both samples were analyzed by formation of the phosphomolybdate complex and its extraction with isobutanol–xylene. A sample of the aqueous phase was counted by the Čerenkov effect or by scintillation counting to determine glucose-6-[$^{32}$P]phosphate alone (boiled samples) or [$\gamma$-$^{32}$P]GTP plus glucose-6-[$^{32}$P]phosphate (nonboiled samples).

### *Analysis of Results*

The results were analyzed with the aid of an Apple II+ minicomputer (48K RAM), using programs written in Applesoft BASIC. Apparent $K_m$ and $V_{max}$ values were calculated by nonlinear least-squares fit to the Michaelis–Menten equation (Roberts, 1977; Duggleby, 1981). Values of $K_{i(slope)}$ and $K_{i(intercept)}$ were estimated with a modified version of the latter program (Duggleby, 1981), from the relations between these parameters and the apparent $K_m$ and $V_{max}$ values (Cornish-Bowden, 1979).

Preliminary estimates of the constants in the more complicated equations were obtained with the aid of a program that allows manual input of values for constants into any equation. The program calculates velocity-versus-substrate concentration curves for several inhibitor concentrations and displays them so as to allow their visual comparison with the experimental results. This

program was also useful for testing the effects on these curves of varying the values of individual constants. The final fitting of all the equations to the total set of results was done with the GRIDLS (grid-search least-squares) subroutine of Bevington (1969). The curves shown in this paper were calculated from parameters determined by this program.

## Results

### *ATP and GTP Synthesis as a Function of Nucleotide Concentrations*

Studies of enzymatic reactions in the presence of two alternative substrates can give positive evidence for a flip-flop mechanism, as discussed in detail elsewhere (Bale *et al.*, 1980). In the present work, we used ADP and GDP as alternative substrates in photophosphorylation, for several reasons. Both ADP and GDP are phosphorylated by chloroplast membranes at high rates, but their apparent $K_m$ values differ significantly from each other (Magnusson and McCarty, 1975; Schlimme *et al.*, 1979; Franek and Strotmann, 1981). In addition, GDP can prevent some apparent regulatory effects of ADP without itself significantly affecting the ATP synthetase (Shavit *et al.*, 1981; Bar-Zvi and Shavit, 1982). Thus, we expected that the basic parameters associated with the phosphorylation of these two compounds might be sufficiently different to allow the proposed mechanisms to be readily distinguished. Finally, of all the possible alternative substrates that could be used, apparently only GDP yields a triphosphate that does not react significantly with hexokinase (Bennun and Avron, 1965; Schlimme *et al.*, 1979). Thus the terminal phosphoryl group of the ATP produced could be continuously transferred to glucose-6-phosphate, both to keep the concentration of ADP relatively constant and to allow quantitative separation and measurement of the phosphorylated products.

We checked the amount of glucose-6-phosphate produced by phosphorylating chloroplasts in the absence of added ADP and the presence or absence of GDP. Calculations, based on the Michaelis–Menten equation and the apparent $K_m$ and $V_{max}$ values determined for ADP in the same experiments, showed that the chloroplasts typically contributed about 0.2 $\mu$M endogenous soluble ADP to the reaction mixtures. (By comparison, most experiments were done with 10–150 $\mu$M added ADP.) In addition, either GDP was contaminated with about 0.3% of ADP, or GTP reacts slightly (2–3%) with hexokinase. The data were not corrected for these factors, because they seemed negligible (see also Segel, 1975, p. 93). The amount of hexokinase used was high enough that an observed inhibition of this enzyme (at low hexokinase concentrations) by GDP did not affect the results. That is, the apparent $K_m$ and $V_{max}$ values for ADP and $K_i$ for GDP were the same at 5 or 10 units of hexokinase/ml, when [ADP] was varied in the presence or absence of GDP (not shown).

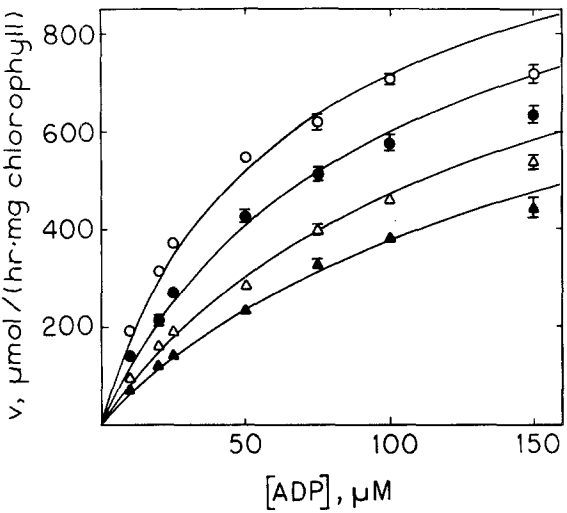


**Fig. 1.** ATP synthesis as a function of ADP concentration at several fixed concentrations of GDP. The experiments were done as described in Materials and Methods. The results of several experiments were normalized with respect to the apparent $V_{max}$, and then the average velocity and standard deviation (when it was larger than the symbol) were plotted for each point. The GDP concentrations were averaged over the duration of the reaction (Segel, 1975, p. 57) and were: (○) none; (●) 240 μM; (△) 730 μM; (▲) 1490 μM. The lines were calculated according to the regulatory-site model, using the values listed in Table III.

Two types of experiments were performed: (a) variation of ADP concentration at several fixed GDP concentrations and vice versa; and (b) change of both [ADP] and [GDP] at several fixed ratios. Each type of experiment was done at least three times. The results for ATP and GTP synthesis that were averaged from several such experiments are shown in Figs. 1–4. Although the results for the two types of experiments are presented separately, all the ATP or GTP results were pooled for analysis by the GRIDLS program. The GDP concentrations were averaged over the duration of the reaction period (Segel, 1975, p. 57), because GDP was not regenerated during the reactions. Usually $\leq$10% of the added GDP was consumed at initial [GDP] $\geq$250 μM, while the amount consumed was <20% at lower GDP concentrations in the absence of competing ADP. The points shown in the figures are the experimental values with their standard deviations, whereas the curves were calculated as described later. As can be seen, the rate of ATP synthesis increases when the nucleotide concentration is raised (Figs. 1 and 3). GDP is a relatively poor inhibitor of ATP synthesis, since the GDP concentration must be higher than that of ADP to obtain even a moderate degree of inhibition. In contrast, ADP

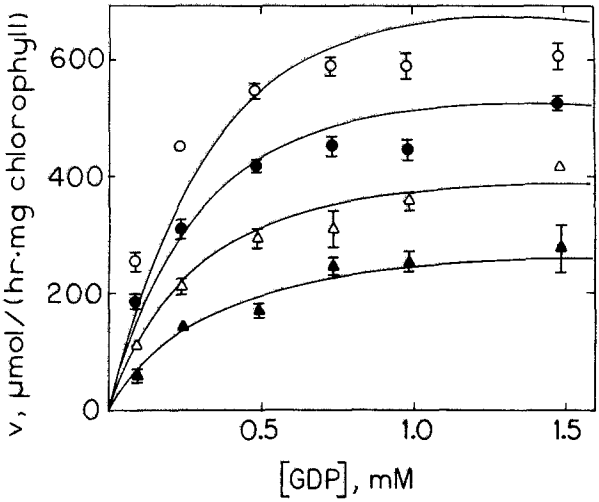


**Fig. 2.** GTP synthesis as a function of GDP concentration at several fixed concentrations of ADP. Calculations were as in Fig. 1. The concentrations of ADP added were: (○) none; (●) 20 $\mu$M; (△) 50 $\mu$M; (▲) 100 $\mu$M.

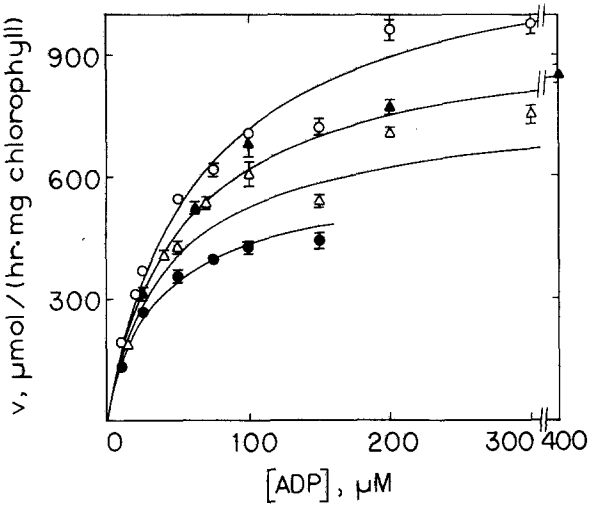


**Fig. 3.** ATP synthesis when the concentrations of both ADP and GDP were varied at several fixed ratios. Calculations were as in Fig. 1. The ratios of [GDP]/[ADP] were: (○) no GDP; (●) 9.79; (△) 4.85; (▲) 1.95.

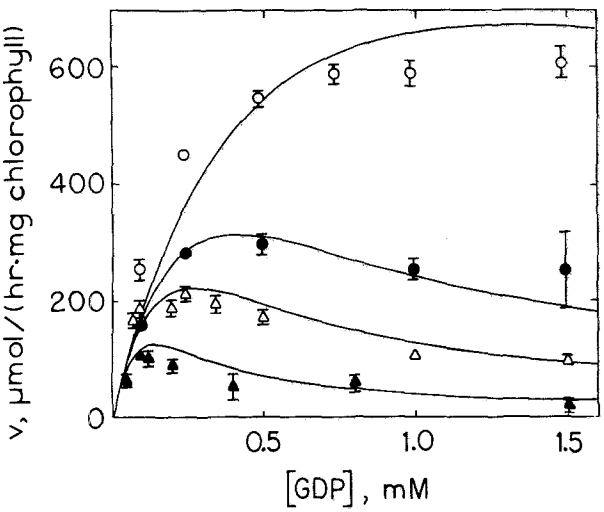


**Fig. 4.** GTP synthesis when the concentrations of both GDP and ADP were varied at several fixed ratios. Calculations were as in Fig. 1. The ratios of [GDP]/[ADP] were: (○) no ADP added; (●) 9.79; (△) 4.85; (▲) 1.95.

is a strong inhibitor of GTP synthesis (Figs. 2 and 4), so much so that the velocity of GTP synthesis actually decreases when the concentrations of both GDP and ADP are increased, even when the concentration of GDP is about 10 times that of ADP (Fig. 4). We tested several models with reference to these results, as presented in the following sections.

### *Mechanism Involving Only Noninteracting Catalytic Sites*

If ADP and GDP simply compete with each other as alternative substrates at a single site or at noninteracting catalytic sites, they should be strictly competitive inhibitors with respect to each other. That is, the slope, but not the intercept, of a double-reciprocal plot should be influenced by the alternative substrate. In addition, the $K_m$ for a substrate should equal its $K_{i(slope)}$ as an inhibitor (Segel, 1975, pp. 790–810; Bale *et al.*, 1980). Since the equation for this (Michaelian) mechanism is just a limiting case of the complete equation for linear mixed-type competition, we fit the latter equation (Segel, 1975, p. 170) to the results shown in Figs. 1–4, using the programs mentioned in Materials and Methods. The final values determined for the parameters are shown in Table I. The effect of GDP on ATP synthesis indeed resembles strict competitive inhibition; however, the $K_{i(slope)}$ for GDP (680 $\mu$M) is very different from the $K_m$ for GDP (350 $\mu$M). In addition, ADP clearly acts like a mixed-type competitor with respect to GTP synthesis, since both $K_{i(slope)}$ and $K_{i(intercept)}$ have finite, well-defined values (Table I). Thus, we

**Table I.** Best-Fit Parameters, Assuming Linear Mixed-Type Competition Between ADP and GDP[a]

| Parameter | Nucleotide | |
|---|---|---|
| | ADP | GDP |
| $V_{max}$, $\mu$mol/(hr · mg chlorophyll) | 1081(10) | 917 (15) |
| $K_m$, $\mu$M | 57.2 (0.8) | 347 (11) |
| $K_{i\ (intercept)}$, $\mu$M | 46.9 (2.5) | —[b] |
| $K_{i\ (slope)}$, $\mu$M | 71.3 (6.2) | 675 (17) |

[a]The parameters (standard deviation in parentheses) were determined from the results shown in Figs. 1 and 3 (ATP) and Figs. 2 and 4 (GTP) with the GRIDLS program. The curves drawn using these parameters (not shown) fit the results rather well, except that the predicted downward curvature for the results shown in Fig. 4 was not extensive enough. The chi square was 3.4 for the ATP results, and 4.0 for the GTP results.

[b]The estimated value for $K_{i(intercept)}$ for GDP was very high ($\simeq$0.1 M) and uncertain. Thus, GDP has no experimentally significant effect on the $V_{max}$ for ATP synthesis.

may conclude that more than one catalytic site is present and that they interact (perhaps by a flip-flop mechanism) and/or that ADP and GDP also compete for noncatalytic, regulatory sites.

### *Pure Flip-Flop Mechanism*

A flip-flop mechanism is defined as one in which the release of product from one catalytic site on an enzyme is stimulated by the binding of substrate at another catalytic site. The methods by which such a mechanism can be distinguished were presented in detail by Bale *et al.* (1980), with reference to the alkaline phosphatase reaction. Since the limiting assumptions used in that study may not apply to the mechanism of photophosphorylation, we derived equations specific for phosphorylation. The reaction mechanism considered here is shown in Fig. 5, where the $k_1$–$k_2$–$k_5$ cycle represents ordinary Michaelian catalysis and the $k_2$–$k_3$–$k_4$ cycle is a mechanism where product release requires prior substrate binding (step 3). As shown, the model assumes that ADP binds before $P_i$ (Selman and Selman-Reimer, 1981), but since the same concentration of $P_i$ was used in all the experiments presented here, the

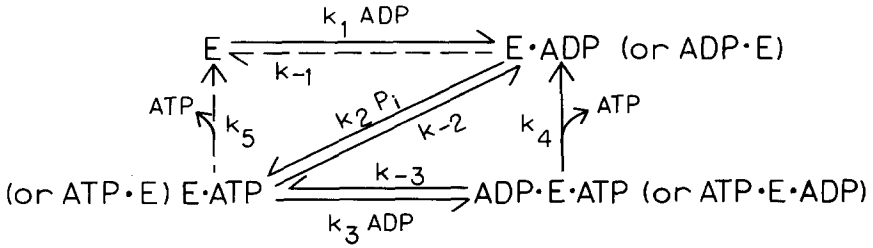


**Fig. 5.** Flip-flop mechanism for phosphorylation of ADP. See text for details.

exact step at which $P_i$ binds does not affect the form of the equations. We also assumed that the concentration of nucleoside triphosphate was low enough that its rebinding was essentially zero. This was accomplished by either immediately transferring the terminal phosphoryl group of ATP to glucose-6-phosphate or by allowing no more than 10–20% of the substrate (when it was GDP) to be consumed.

A steady-state analysis by the King–Altman method (Segel, 1975, Chapter 9) yields the following equation:

$$v = \frac{K1[\mathrm{ADP}] + K2[\mathrm{ADP}]^2}{K3 + K4[\mathrm{ADP}] + K5[\mathrm{ADP}]^2} \tag{1}$$

where $K1$–$K5$ are various combinations of rate constants and $[P_i]$. The rate of photophosphorylation as a function of ADP or GDP concentration appears to be a simple hyperbolic function at least at low nucleoside diphosphate concentrations (e.g., Bennun and Avron, 1965; Magnusson and McCarty, 1975; Selman and Selman-Reimer, 1981), and classical flip-flop enzymes are supposed to exhibit Michaelian kinetics (see Bale *et al.*, 1980), but Eq. (1) is not a simple Michaelis–Menten function. However, it reduces to Michaelian form if $K1$ and $K3 = 0$. Since $K1 = k_1k_2k_5(k_{-3} + k_4)[P_i][E_t]$, $K2 = k_1k_2k_3k_4[P_i][E_t]$, and $K3 = (k_5(k_{-1} + k_2[P_i]) + k_{-1}k_{-2}) \times (k_{-3} + k_4)$, $K1$ will equal zero if $k_5$ is zero, i.e., if release of product absolutely depends on binding of substrate to another catalytic site. The value of $K3$ will be zero if $k_5$ and either $k_{-1}$ or $k_{-2} = 0$. It seems more reasonable to assume in this case that $k_{-1} = 0$, i.e., that the catalytic site always contains a nucleotide under phosphorylating conditions (Rosen *et al.*, 1979; Boyer and Kohlbrenner, 1981). If $K1$ and $K3 = 0$, then one [ADP] term cancels out, and $V_{max} = K2/K5$ while $K_m = K4/K5$.

Studies of reaction velocity in the presence of two alternative substrates can yield valuable evidence on the enzyme mechanism (see Bale *et al.*, 1980). A complete model for the simultaneous phosphorylation of ADP and GDP by flip-flop mechanisms is shown in Fig. 6. Here, the $k_1$–$k_5$ steps are the same as in Fig. 5, and the $k_{11}$–$k_{15}$ steps represent the same reactions when GDP is the substrate. The $k'$ steps are numbered analogously, but refer to the enzyme forms containing both one product and the alternative substrate. Assuming as before that $[P_i]$ is constant, that $[\mathrm{NTP}] \simeq 0$, and that the rate constants for all reaction steps leading directly toward free E are zero (indicated by dashed lines in the figure), a complete steady-state analysis yields the following equations for the rates of ATP and GTP synthesis.

$$v_{\mathrm{ATP}} = \frac{K6[\mathrm{ADP}]^2 + K7[\mathrm{ADP}][\mathrm{GDP}]}{K8[\mathrm{ADP}] + K9[\mathrm{ADP}]^2 + K10[\mathrm{GDP}] + K11[\mathrm{GDP}]^2 + K12[\mathrm{ADP}][\mathrm{GDP}]} \tag{2}$$

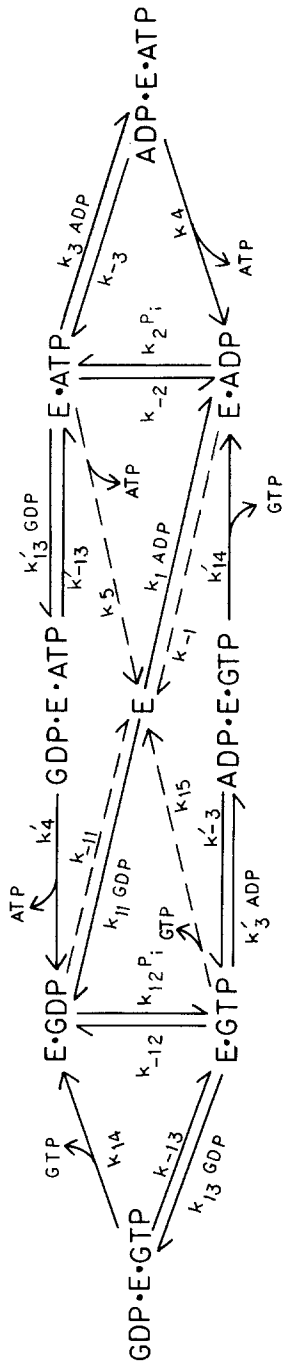


**Fig. 6.** Phosphorylation of GDP and ADP simultaneously, by pure flip-flop mechanism.

$$v_{GTP} = \frac{K13[GDP]^2 + K14[ADP][GDP]}{K8[ADP] + K9[ADP]^2 + K10[GDP] + K11[GDP]^2 + K12[ADP][GDP]} \quad (3)$$

These equations do not describe simple (Michaelian) hyperbolas, so that plots of $1/v$ versus $1/[S]$ should not be strictly linear when the concentration of one substrate is varied in the presence of several fixed concentrations of the other. However, these equations predict that double-reciprocal plots must be linear when the concentrations of both substrates are varied such that [ADP] = ([GDP] multiplied by a constant). This is clearly inconsistent with the experimental results shown in Fig. 4, since the lines in a double-reciprocal plot of these results curve upward as they approach the $y$ axis. Thus, a pure flip-flop mechanism seems impossible for photophosphorylation.

If the rate constants for release of NDP ($k_{-1}$ and $k_{-11}$) and/or those for release of NTP in the absence of bound NDP ($k_5$ and $k_{15}$) do not equal zero, the velocity equations would be more complex than Eqs. (2) and (3). However, the equations for ATP and GTP synthesis would both be of the same form, with identical constants in the denominators. Trials of various values for $K6$–$K14$ in Eqs. (2) and (3) (using the manual-input program described in Materials and Methods) revealed that these two mathematical requirements represent serious limitations, so that it is doubtful whether even such a mechanism, which is only a loose flip-flop, could fit the results.

### *Selective Flip-Flop Mechanism*

Studies with inhibitors have suggested that ADP and GDP might not be phosphorylated in exactly the same way (Magnusson and McCarty, 1975). In addition, GDP has a weaker apparent regulatory effect on $CF_1$ function than does ADP (Shavit *et al.*, 1981; Strotmann *et al.*, 1981a; Shavit, 1980). Since the pure flip-flop mechanism did not fit our results, we therefore also considered the possibility that the ATP-synthesizing complex might handle ADP by a flip-flop mechanism and GDP in a simple Michaelian manner. That is, ATP synthesis would occur with significant, required, alternating conformational changes, while GTP synthesis would not. Consequently, the enzyme does not "know" whether or not the alternative catalytic site is occupied by GDP. GTP release normally occurs from the enzyme form that does not have ADP present in the alternative site, while ATP release requires the ADP-induced enzyme conformation.

Such a mechanism is shown in Fig. 7. Here, the ADP phosphorylation steps associated with the constants $k_1$–$k_5$ are the same as in Figs. 5 and 6. The $k_{11}$–$k_{12}$–$k_{15}$ cycle represents a simple Michaelian mechanism for GTP synthesis. In addition, GDP can bind to the E · ATP form ($k'_{13}$) without altering the enzyme conformation, and ADP can bind to the E · GTP form ($k'_{14}$). In this

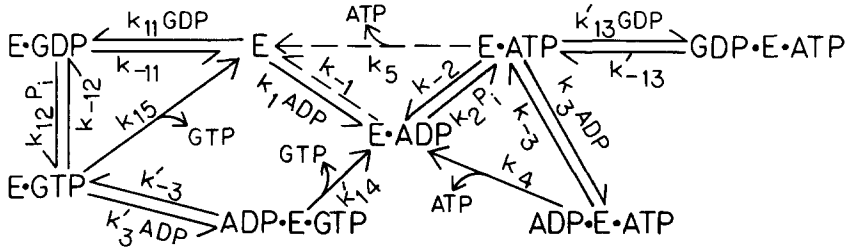


**Fig. 7.** Phosphorylation of ADP by flip-flop mechanism and of GDP by simple mechanism.

mechanism, if $k_{-1}$ and $k_5 = 0$ as before, then GDP would be a simple competitive inhibitor with respect to ATP synthesis, with its $K_{i(slope)}$ not necessarily equal to the $K_m$ for GDP as a substrate. Such results were obtained (Table I). However, ADP would be an infinitely powerful inhibitor of GTP synthesis, since under initial velocity conditions the pathway toward E · ADP from the E · guanosine nucleotide forms is unidirectional. Since we found that GTP synthesis does occur in the presence of ADP (Figs. 2 and 4), we must assume in this case that $k_{-1}$ does not equal zero. A steady-state analysis then yields the following equations for the rates of ATP and GTP synthesis.

$$v_{ATP} = \frac{K15[ADP]^2 + K16[ADP]^3 + K17[ADP]^2[GDP]}{K21 + K22[ADP] + K23[ADP]^2 + K24[ADP]^3 + K25[GDP] + K26[ADP][GDP] + K27[ADP]^2[GDP] + K28[GDP]^2[ADP]} \quad (4)$$

$$v_{GTP} = \frac{K18[GDP] + K19[ADP][GDP] + K20[ADP]^2[GDP]}{K21 + K22[ADP] + K23[ADP]^2 + K24[ADP]^3 + K25[GDP] + K26[ADP][GDP] + K27[ADP]^2[GDP] + K28[GDP]^2[ADP]} \quad (5)$$

Note that now it is possible for the effects of GDP on ATP synthesis to be qualitatively different from the effects of ADP on GTP synthesis, since the two numerators are not of the same form.

Table II shows the parameters obtained by using the GRIDLS program to fit this model to our results. The model appears to be invalid for the following reasons: several of the best-fit parameters are significantly less than zero (Table II), which is kinetically impossible; calculations of the ratio $K18/K15$ from the appropriate pairs of values (Table II) yield values differing by over three orders of magnitude; and the differences between the actual reaction velocities and velocities calculated from these parameters and Eqs. (4) or (5) were obviously correlated with the concentrations of substrates (not shown). Even when we used the manual fitting program to ensure identical values for

**Table II.** Best-Fit Parameters for Selective Flip-Flop Model[a]

| ATP results | | GTP results | |
|---|---|---|---|
| Parameter | Value (s.d.) | Parameter | Value (s.d.) |
| $K16/K15$ | 0.0140 (0.0003) | $K19/K18$ | 1.92 (0.14) |
| $K17/K15$ | 0.00193 (0.00002) | $K20/K18$ | −0.00233 (0.00159) |
| $K21/K15$ | 0.211 (0.027) | $K21/K18$ | 3.90 (0.29) |
| $K22/K15$ | 0.0301 (0.0020) | $K22/K18$ | 0.840 (0.169) |
| $K23/K15$ | 0.00191 (0.00006) | $K23/K18$ | −0.0111 (0.0035) |
| $K24/K15$ | $1.13\ (0.05) \times 10^{-5}$ | $K24/K18$ | $2.03\ (0.36) \times 10^{-4}$ |
| $K25/K15$ | −0.00145 (0.00018) | $K25/K18$ | −0.0394 (0.0025) |
| $K26/K15$ | $3.43\ (0.07) \times 10^{-4}$ | $K26/K18$ | 0.00474 (0.00054) |
| $K27/K15$ | $2.69\ (0.09) \times 10^{-6}$ | $K27/K18$ | $4.29\ (9.73) \times 10^{-6}$ |
| $K28/K15$ | $6.87\ (0.53) \times 10^{-8}$ | $K28/K18$ | $-6.93\ (9.82) \times 10^{-8}$ |
| Chi square | 2.2 | Chi square | 79 |

[a] The results shown in Figs. 1 and 3 (ATP) and Figs. 2 and 4 (GTP) were fitted to Eqs. (4) and (5), respectively, using the GRIDLS program. The numerator and denominator of each equation were divided by $K15$ or $K18$, respectively, to obtain unique fits. The curves calculated from these values (not shown) predicted negative velocity for GTP synthesis in the absence of ADP.

identical parameters and positive values for all parameters, this model allowed for the downward curvature shown in Fig. 4 only if ADP stimulated rather than inhibited GTP synthesis, which is clearly not what occurs. Thus a model that allows for flip-flop phosphorylation of ADP and simple phosphorylation of GDP also cannot be valid, in spite of its potential for explaining why ADP and GDP appear to interact differently with the tight-binding sites of chloroplast $CF_1$ (e.g., Magnusson and McCarty, 1975; Bar-Zvi and Shavit, 1982). We did not consider what might happen if $k_5$ does not equal zero, since this would no longer be a flip-flop mechanism, because the release of ATP would not require an enzyme conformational change such as that induced by ADP binding.

### *Noncatalytic Regulatory Site Mechanism*

Because a ligand need not be repeatedly bound and released at a noncatalytic regulatory site during each catalytic cycle, and in order to prevent the analysis from becoming nearly impossibly complicated, we assumed that binding to the regulatory site(s) could be treated as an equilibrium (rather than a steady-state) process. Since the time course for ATP synthesis is linear under our conditions (not shown), this implies that equilibration of nucleotides with the regulatory site occurs prior to or concomitant with the establishment of the steady-state rate of ATP synthesis (see also Schlodder and Witt, 1981), which in turn implies that regulatory ADP binds primarily to enzyme having unoccupied catalytic site(s). We also assume that any regulatory nucleotide is the diphosphate under our condi-

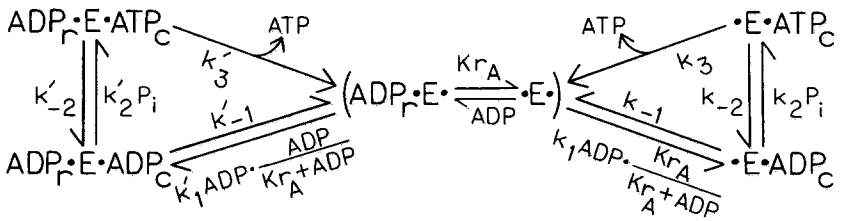


**Fig. 8.** A regulatory-site model for ATP synthesis. The subscripts r and c denote nucleotides located in the regulatory and catalytic sites, respectively, and $K_{r_A}/(K_{r_A} + [ADP])$ and $[ADP]/(K_{r_A} + [ADP])$ represent the fractions of E · with free and occupied regulatory sites, respectively.

tions, since the concentrations of nucleoside triphosphates are much lower. Such a mechanism is shown in Fig. 8. This figure represents simple Michaelian catalysis of ATP synthesis by two different forms of the enzyme, with the equilibrium between the two forms being determined by the ADP concentration and the equilibrium constant ($K_{r_A}$) for ADP binding to the regulatory site. A combined equilibrium and steady-state analysis by the method of Cha (Segel, 1975; Cornish-Bowden, 1979) yields the following equation for the velocity of ATP synthesis as a function of [ADP] when ADP is the only nucleoside diphosphate present:

$$v_{ATP} = \frac{V_{max_A}[ADP](\alpha K_{r_A} + \beta\,[ADP])}{\alpha K_{m_A}(K_{r_A} + [ADP]) + [ADP](\alpha K_{r_A} + [ADP])} \tag{6}$$

Here, $K_{r_A}$ is the equilibrium dissociation constant for ADP at the regulatory site, and $K_{m_A}$ is the Michaelis constant for the catalytic site (defined as in Segel, 1975, p. 524). The constant $\alpha$ represents the effect of ADP in the regulatory site on the binding of ADP to the catalytic site (and necessarily vice versa, since binding of ADP to the catalytic site of ·E decreases the concentration of the enzyme form that can bind ADP in its regulatory site), and $\beta$ is the effect of the regulatory site on the reaction velocity. The constants as defined here are apparent constants inasmuch as they depend on the $P_i$ concentration. Equation (7) is that for GTP synthesis in the absence of ADP, where $\alpha°$ and $\beta°$ are the $\alpha$ and $\beta$ constants associated with GDP.

$$v_{GTP} = \frac{V_{max_G}[GDP](\alpha° K_{r_G} + \beta°[GDP])}{\alpha° K_{m_G}(K_{r_G} + [GDP]) + [GDP](\alpha° K_{r_G} + [GDP])} \tag{7}$$

When both ADP and GDP are present in the reaction mixture, the regulatory site can contain ADP or GDP or be empty. Thus, there are three different forms of the enzyme, $ADP_r$ · E, $GDP_r$ · E, and · E, each capable of binding either GDP or ADP at the catalytic site (not shown in figure). The velocity equation for ATP synthesis is the following:

$$v_{ATP} = \frac{V_{max_A}[ADP](\alpha K_{r_A}(\delta K_{r_G} + \epsilon[GDP]) + \beta[ADP]\delta K_{r_G})}{\begin{aligned}\alpha K_{m_A}(K_{r_A}(\delta K_{r_G}(1 + [GDP]/K_{m_G}) + \delta[GDP](1 + [GDP]/\alpha^\circ K_{m_G})) \\ + [ADP](\delta K_{r_G}(1 + [GDP]/\delta^\circ K_{m_G}))) \\ + [ADP](\alpha K_{r_A}(\delta K_{r_G} + [GDP]) + [ADP]\delta K_{r_G})\end{aligned}} \quad (8)$$

This is like Eq. (6), except the ADP constants are all multiplied by various constants associated with GDP. In Eq. (8), $\epsilon$ represents the effect of GDP in the regulatory site on the reaction velocity, and $\delta$ and $\delta^\circ$ are the effects of GDP on ADP binding and vice versa. The equation for GTP synthesis (not shown) is analogous to Eq. (8).

Equation (8) and the analogous equation for GTP synthesis were fitted to the results shown in Figs. 1–4, using the GRIDLS program. The various parameters had to be grouped in order to obtain unique fits, and the estimates for these grouped parameters are shown in Table III. The velocity curves

**Table III.** Parameters for Regulatory Site Model[a]

| ATP results | |
|---|---|
| Parameter | Value[b] (s.d.) |
| $\beta/\alpha K_{r_A}$ | 0.0175 (0.0004) |
| $\epsilon/\delta K_{r_G}$ | 0.00133 (0.00003) |
| $K_{m_A}/V_{max_A}$ | 0.0468 (0.0022) |
| $K_{m_A}((1/K_{m_G}) + (1/K_{r_G}))/V_{max_A}$ | $2.06\ (0.07) \times 10^{-4}$ |
| $K_{mA}/\alpha^\circ K_{m_G} K_{r_G} V_{max_A}$ | $3.92\ (0.51) \times 10^{-8}$ |
| $K_{m_A}((1/K_{m_A}) + (1/K_{r_A}))/V_{max_A}$ | 0.00193 (0.00007) |
| $1/V_{max_A}\alpha K_{r_A}$ | $1.43\ (0.06) \times 10^{-5}$ |
| $((K_{m_A}/\delta^\circ K_{m_G} K_{r_A}) + (1/\delta K_{r_G}))/V_{max_A}$ | $3.17\ (0.09) \times 10^{-6}$ |
| Chi square | 2.7 |

| GTP results | | |
|---|---|---|
| Parameter | Value (s.d.) | Values[b] (s.d.) |
| $\beta^\circ/\alpha^\circ K_{r_G}$ | $2.00\ (0.19) \times 10^{-4}$ | $2.69\ (2.08) \times 10^{-5}$ |
| $\epsilon^\circ/\delta^\circ K_{r_A}$ | −0.00225 (0.00014) | 0[c] |
| $K_{m_G}/V_{max_G}$ | 0.440 (0.013) | 0.462 (0.015) |
| $K_{m_G}((1/K_{m_A}) + (1/K_{r_A}))/V_{max_G}$ | 0.00252 (0.00031) | 0.00113 (0.00042) |
| $K_{m_G}/\alpha K_{m_A} K_{r_A} V_{max_G}$ | $5.86\ (3.08) \times 10^{-6}$ | $4.97\ (0.44) \times 10^{-5}$ |
| $K_{m_G}((1/K_{m_G}) + (1/K_{r_G}))/V_{max_G}$ | $8.49\ (0.45) \times 10^{-4}$ | $7.73\ (0.48) \times 10^{-4}$ |
| $1/V_{max_G}\alpha^\circ K_{r_G}$ | $5.65\ (0.48) \times 10^{-7}$ | $3.14\ (0.49) \times 10^{-7}$ |
| $((K_{m_G}/\delta K_{m_A} K_{r_G}) + (1/\delta^\circ K_{r_A}))/V_{max_G}$ | $1.81\ (0.08) \times 10^{-5}$ | $2.03\ (0.12) \times 10^{-5}$ |
| Chi square | 2.4 | 3.2 |

[a] In order to fit this model with the GRIDLS program, Eq. (8) was converted to the form $([ADP] + A[ADP]^2 + B[ADP][GDP])/(C + D[GDP] + E[GDP]^2 + F[ADP] + G[ADP]^2 + H[ADP][GDP])$ by grouping the constants and dividing the numerator and denominator by $V_{max_A}\alpha K_{r_A}\delta K_{r_G}$. The equation for GTP synthesis was treated analogously.

[b] The curves calculated from these parameters are shown in Figs. 1–4.

[c] The equation was refitted to the results, after assuming that $\epsilon^\circ/\delta^\circ K_{r_A}$ should be zero rather than negative.

calculated from these values are shown in Figs. 1–4. In contrast to the flip-flop models, the calculated curves were able to fit both the ATP and GTP results. The fit to the GTP results was good (chi square = 2.4) when we included the parameter $\epsilon^\circ$ in the equation, whose best-fit value was evidently negative. On the assumption that this constant should actually equal zero, i.e., that ADP in the regulatory site completely inhibits the rate of GTP synthesis, we refitted the GTP results to the regulatory site model, and the resulting chi square was not much worse (3.2). The values determined assuming that $\epsilon^\circ = 0$ were used to calculate the curves shown in Figs. 2 and 4. The values for individual parameters cannot be obtained from the values shown in Table III; however, the ratio $K_{m_G} V_{max_A} / K_{m_A} V_{max_G}$ can be calculated from several of the relationships (Table III), and the values obtained for this ratio, while not identical, are all of the same order of magnitude.

## Discussion

In summary, the results of experiments in which chloroplasts simultaneously synthesized ATP and GTP appear to rule out a flip-flop mechanism for photophosphorylation, because a pure flip-flop mechanism is mathematically incapable of fitting these results, while a modified (selective) flip-flop mechanism yields velocity curves which clearly do not fit the results. It makes no difference whether the number of alternating sites is two (Smith and Boyer, 1976; Rosen *et al.*, 1979) or three (Boyer and Kohlbrenner, 1981) (so long as only two catalytic sites are occupied simultaneously) since the essential feature in either case is the concerted binding of substrate at one catalytic site and release of product at one other catalytic site. If such a three-site model were correct, additional central complexes would be added in the reaction sequence shown in Fig. 5, which would not affect the final form of the steady-state velocity equation (Segel, 1975, p. 515).

Similarly, models involving either a very tightly bound ADP functioning as a phosphorylated intermediate (Roy and Moudrianakis, 1971; Tiefert and Moudrianakis, 1979), or an obligatory transfer of ligands between the medium and an always-buried catalytic site (Kozlov and Skulachev, 1977), also result in steady-state velocity equations that are indistinguishable from those for a simple Michaelis–Menten mechanism, or, depending on the overall details of the model, for a flip-flop mechanism. Thus, provided these models truly allow for no "slippage" (that is, that more steps in sequence, but no branch pathways, are added to the simpler models), the present results would also rule out these models for photophosphorylation.

In contrast, the velocity equations derived for the particular model involving noncatalytic regulatory sites that we tested were able to fit all the

results obtained here relatively well. This does not, of course, prove that it is the correct mechanism. For example, we did not consider a model where ADP might be able to bind to the regulatory site no matter what ligands are present in the catalytic site. Such a model is much more complicated than that shown in Fig. 8, and it yields a velocity equation containing sets of rate constants that cannot be defined in terms of $K_m$, $V_{max}$, etc. We also did not analyze a model where stimulatory ATP and inhibitory ADP compete for a very high-affinity regulatory site (Bar-Zvi and Shavit, 1980), or models involving multiple regulatory and/or catalytic sites, or combining noncatalytic regulatory sites with a flip-flop catalytic mechanism. The finding that our results do not fit the tested regulatory model perfectly (Figs. 1–4; Table III) implies that one or more of the above considerations may result in an improvement over the basic model. However, all result in much more complicated reaction schemes which may not yield distinguishable velocity equations. The same may be said for the most recent version of the binding-change mechanism (Gresser *et al.*, 1982), in which three nucleotides can be bound to the enzyme at once, and which seems no longer equivalent to a flip-flop mechanism. Thus, we agree with Boyer that a two-site alternating mechanism cannot account for ATP synthesis. We feel that the regulatory model analyzed here may represent a first approximation of the actual mechanism for photophosphorylation.

Use of the GRIDLS program required that constants in the regulatory site model be grouped, but it was possible to estimate values for some of the individual parameters, after assuming that $\alpha K_{r_A} \simeq 4\ \mu M$ under photophosphorylating conditions (Strotmann *et al.*, 1981a). Several of the determined values were consistent with results of other studies. For example, the binding of ADP to a noncatalytic site is associated with inhibition of the activities of membrane-bound $CF_1$ (Shavit, 1980; Bar-Zvi and Shavit, 1980; Dunham and Selman, 1981; Schumann and Strotmann, 1981; Strotmann *et al.*, 1981a; Schlodder and Witt, 1981; Strotmann *et al.*, 1979). Decreasing the light intensity, which leads to more tight binding of ADP (Strotmann *et al.*, 1981a, b), also results in a decrease in both the apparent $V_{max}$ and apparent $K_m$ for ADP at the catalytic site (Magnusson and McCarty, 1975; Bickel-Sandkötter and Strotmann, 1981; Vinkler, 1981). Here, the regulatory-site equations fit best when $\alpha \simeq 0.16$ and $\beta \simeq 0.07$; that is, when ADP in the regulatory site decreases both the apparent $V_{max}$ ($\rightarrow \beta V_{max}$) and $K_m$ ($\rightarrow \alpha K_m$) for the catalytic site. In addition, here we find that the equations fit best when $\alpha^{\circ} K_{r_G}$ (the effective binding constant for GDP, 2.3 mM) is much higher than $\alpha K_{r_A}$ (4 $\mu$M). This is consistent with the finding that the apparent $K_d$ for GDP binding to the regulatory site is much higher than that for ADP (Bar-Zvi and Shavit, 1982).

An important test of the regulatory-site model presented here will be its ability to explain other types of results. For instance, studies of ATP synthesis

in mitochondria showed that the addition of certain ADP analogs caused the velocity-versus-ADP concentration curves to become sigmoidal (Schäfer and Onur, 1980). While using the manual-fitting program for Eq. (8), we noticed that the curves for ATP synthesis in the presence of fixed amounts of GDP were sigmoidal when the value of $\delta^\circ$ was too high relative to those of the other constants. Whether our model can quantitatively account for these results would require further experimental work. In addition, pretreating chloroplasts with *N*-ethylmaleimide or including 4′-deoxyphlorizin in the assay mixture for photophosphorylation affects both the apparent $K_m$ and $V_{max}$ for ADP, but only the apparent $K_m$ for GDP (Magnusson and McCarty, 1975). Over the range of substrate concentrations used in that work, and according to the estimated values for $\alpha K_{T_A}$ and $\alpha^\circ K_{T_G}$, the major form of $CF_1$ present during GTP synthesis would have unoccupied regulatory sites. During ATP synthesis, the main form would be $CF_1$ with ADP in the regulatory sites. If these two forms of the enzyme have different sensitivities to inhibitors, this regulatory-site mechanism could explain these results. Finally, changing the concentration of $P_i$ at which ATP synthesis is assayed changes the pattern of competition between ADP and the inhibitor $\beta$-thioADP (Selman and Selman-Reimer, 1981). Study of the dependence of the ADP kinetic patterns on $[P_i]$ will require the derivation and testing of the complete velocity equation specifically including $[P_i]$ and the true kinetic constants and regulatory factors associated with both ADP and $P_i$. The findings here, that the effect of GDP on ATP synthesis resembles competitive inhibition, while the effect of ADP on GTP synthesis resembles mixed-type ("noncompetitive") inhibition (Table I), suggest that the expanded model probably could accommodate such results.

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## References

Aflalo, C., and Shavit, N. (1982). Source of rapidly labeled ATP tightly bound to noncatalytic sites on the chloroplast ATP synthetase, *Eur. J. Biochem.* **126**, 61–68.

Bale, J. R., Boon Chock, P., and Huang, C. Y. (1980). The nature of negative cooperativity in alkaline phosphatase. Kinetic patterns contrary to the flip-flop model, *J. Biol. Chem.* **255,** 8424–8430.

Bar-Zvi, D., and Shavit, N. (1980). Role of tight nucleotide binding in the regulation of the chloroplast ATP synthetase activities, *FEBS Lett.* **119,** 68–72.

Bar-Zvi, D., and Shavit, N. (1982). Modulation of the chloroplast ATPase by tight binding of nucleotides, *Biochim. Biophys. Acta* **681,** 451–458.

Bennun, A., and Avron, M. (1965). The relation of the light-dependent and light-triggered adenosine triphosphatases to photophosphorylation, *Biochim. Biophys. Acta* **109,** 117–127.

Bevington, P. R. (1969). *Data Reduction and Error Analysis for the Physical Sciences,* McGraw-Hill, New York, pp. 212–213.

Bickel-Sandkötter, S., and Strotmann, H. (1981). Nucleotide binding and regulation of chloroplast ATP synthase, *FEBS Lett.* **125,** 188–192.

Boyer, P. D., and Kohlbrenner, W. E. (1981). The present status of the binding-change mechanism and its relation to ATP formation by chloroplasts, in: *Energy Coupling in Photosynthesis* (Selman, B. R., and Selman-Reimer, S., eds.), Elsevier/North-Holland, New York, pp. 231–240.

Cornish-Bowden, A. (1979). *Fundamentals of Enzyme Kinetics,* Butterworths, London.

Duggleby, R. G. (1981). A nonlinear regression program for small computers, *Anal. Biochem.* **110,** 9–18.

Dunham, K. R., and Selman, B. R. (1981). Regulation of spinach chloroplast coupling factor 1 ATPase activity, *J. Biol. Chem.* **256,** 212–218.

Franek, U., and Strotmann, H. (1981). Nucleotide specificity of $CF_1$-ATPase in ATP synthesis and ATP hydrolysis, *FEBS Lett.* **126,** 5–8.

Gresser, M. J., Myers, J. A., and Boyer, P. D. (1982). Catalytic cooperativity of beef heart mitochondrial $F_1$ adenosine triphosphatase. Correlations of initial velocity, bound intermediate, and oxygen exchange measurements with an alternating three-site model, *J. Biol. Chem.* **257,** 12030–12038.

Kozlov, I. A., and Skulachev, V. P. (1979). $H^+$-adenosine triphosphatase and membrane energy coupling, *Biochim. Biophys. Acta* **463,** 29–89.

Magnusson, R. P., and McCarty, R. E. (1975). Influence of adenine nucleotides on the inhibition of photophosphorylation in spinach chloroplasts by *N*-ethylmaleimide, *J. Biol. Chem.* **250,** 2593–2598.

Roberts, D. V. (1977). *Enzyme Kinetics,* Cambridge University Press, Cambridge, pp. 299–306.

Rosen, G., Gresser, M., Vinkler, C., and Boyer, P. D. (1979). Assessment of total catalytic sites and the nature of bound nucleotide participation in photophosphorylation, *J. Biol. Chem.* **254,** 10654–10661.

Roy, H., and Moudrianakis, E. N. (1971). Synthesis and discharge of the coupling factor · adenosine diphosphate complex in spinach chloroplast lamellae, *Proc. Natl. Acad. Sci. U.S.A.* **68,** 2720–2724.

Schäfer, G., and Onur, G. (1980). Unmasking cooperativity of oxidative phosphorylation by a new $\alpha$-phosphate acylated ADP-analog, *FEBS Lett.* **117,** 269–272.

Schlimme, E., deGroot, E. J., Schott, E., Strotmann, H., and Edelmann, K. (1979). Photophosphorylation of base-modified nucleotide analogs by spinach chloroplasts, *FEBS Lett.* **106,** 251–256.

Schlodder, E., and Witt, H. T. (1981). Relation between the initial kinetics of ATP synthesis and of conformational changes in the chloroplast ATPase studied by electric field pulses, *Biochim. Biophys. Acta* **635,** 571–584.

Schumann, J., and Strotmann, H. (1981). The mechanism of induction and deactivation of light-triggered ATPase, in: *Proc. 5th Intern. Congr. Photosynth.* (Akoyunoglou, G., ed.), Vol. 2, Balaban Intern. Sci. Serv., Philadelphia, pp. 881–892.

Segel, I. H. (1975). *Enzyme Kinetics. Behavior and Analysis of Rapid-Equilibrium and Steady-State Enzyme Systems,* Wiley-Interscience, New York.

Selman, B. R., and Selman-Reimer, S. (1981). The steady-state kinetics of photophosphorylation, *J. Biol. Chem.* **256,** 1722–1726.

Shavit, N. (1980). Energy transduction in chloroplasts: Structure and function of the ATPase complex, *Annu. Rev. Biochem.* **49,** 111–138.

Shavit, N., Aflalo, C., and Bar-Zvi, D. (1981). Role of tight nucleotide binding sites in the modulation of the chloroplast ATP synthetase activity, in: *Energy Coupling in Photosynthesis* (Selman, B. R., and Selman-Reimer, S., eds.), Elsevier/North-Holland, New York, pp. 197–207.

Shavit, N., Lien, S., and San Pietro, A. (1977). On the role of membrane-bound ADP and ATP in photophosphorylation in chloroplast membranes, *FEBS Lett.* **73,** 55–58.

Smith, D. J., and Boyer, P. D. (1976). Demonstration of a transitory tight binding of ATP and of committed $P_i$ and ADP during ATP synthesis by chloroplasts, *Proc. Natl. Acad. Sci. U.S.A.* **73,** 4314–4318.

Strotmann, H., Bickel-Sandkötter, S., Edelmann, K., Eckstein, F., Schlimme, E., Boos, K. S., and Lüstorff, J. (1979). Thiophosphate analogs of ADP and ATP as substrates in partial reactions of energy conversion in chloroplasts, *Biochim. Biophys. Acta* **545,** 122–130.

Strotmann, H., Bickel-Sandkötter, S., Franek, U., and Gerke, V. (1981a). Nucleotide interactions with membrane-bound $CF_1$, in: *Energy Coupling in Photosynthesis* (Selman, B. R., and Selman-Reimer, S., eds.), Elsevier/North-Holland, New York, pp. 187–196.

Strotmann, H., Bickel-Sandkötter, S., and Shoshan, V. (1981b). Kinetic analysis of light-dependent exchange of adenine nucleotides in chloroplast coupling factor $CF_1$, *FEBS Lett.* **101,** 316–320.

Tiefert, M. A., and Moudrianakis, E. N. (1979). Role of AMP in photophosphorylation by spinach chloroplasts, *J. Biol. Chem.* **254,** 9500–9508.

Vinkler, C. (1981). Opposite modulation by uncoupling and electron transport limitation of the $K_m^{app}$ of ADP for photophosphorylation, *Biochem. Biophys. Res. Commun.* **99,** 1095–1100.