Studies on the Mechanism of Oxidative Phosphorylation

POSITIVE COOPERATIVITY IN ATP SYNTHESIS

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Kinetic and nucleotide binding studies have shown that submembrane particles from bovine heart possess three exchangeable binding sites for ADP or GDP. In order of decreasing affinity at neutral pH, these sites will be referred to as sites I, II, and III, and their respective dissociation constants as $K_I$, $K_{II}$, and $K_{III}$. In oxidative phosphorylation experiments in the presence of saturating amounts of inorganic phosphate, rapid ATP (or GTP) synthesis occurred only upon ADP (or GDP) binding to site III. The Eadie-Hofstee plots ($v/[S]$ on the ordinate versus $v$ on the abscissa) of the kinetics of ATP (or GTP) synthesis at variable ADP (or GDP) were, therefore, composed of an initial upward phase, indicating positive cooperativity with respect to substrate concentration, followed by a downward phase where rapid product formation took place. These data allowed calculation of plots of binding data with radiolabeled ADP or GDP. Thus, together with our previous results, these findings have allowed characterization of the process of ATP or GTP synthesis by bovine-heart submembrane particles in terms of $K_I$, $K_{II}$, $K_{III}$, and $k_{cat}$.

During the past decade, research on the mechanism of ATP synthesis in oxidative and photosynthetic phosphorylation has been devoted mainly to study of the details of ATP hydrolysis by isolated F$_1$-ATPases. The advantages offered by F$_1$, including its water solubility and relative structural simplicity, and the ease and precision with which the hydrolytic reaction catalyzed by F$_1$ can be monitored, manipulated, and reproduced, have allowed investigators to accumulate a vast amount of valuable information regarding the mechanism of ATP hydrolysis. Several major findings that are relevant to the subject matter of this article are summarized below.

(i) Stereochemical study of the $\gamma$-phosphoryl oxygens of ATP hydrolyzed by bovine heart F$_1$ has shown inversion of chirality; thus, indicating that ATP hydrolysis (or synthesis) by F$_1$ does not involve an X$-$F$_1$ intermediate (1). (ii) At [ATP] $\leq$ [F$_1$], ATP binds tightly ($K_a = 10^{-12}$ M) to only one of the three potential catalytic sites of F$_1$, and is partially and very slowly hydrolyzed (unisite ATP hydrolysis) with turnover number $= 4 \times 10^{-3}$ s$^{-1}$ for the bovine heart F$_1$ (2). Under these conditions, the equilibrium constant for F$_1$-bound substrate and products was shown to be close to unity, thus indicating that conversion of ATP to ADP and P$_i$ on the surface of F$_1$ involved little free energy change (2). This implied that F$_1$ could also catalyze bound ATP synthesis from bound ADP and P$_i$ and was soon demonstrated to be the case with isolated F$_1$ from diverse sources (3-5). These data corroborated earlier predictions of Boyer and his colleagues (6-9) based on the results of SMP-catalyzed P$_i = \text{H}_2\text{O}$ oxygen exchange experiments in the absence and presence of uncouplers. (iii) At [ATP] $\gg$ [F$_1$], the kinetics of ATP hydrolysis were shown to display negative cooperativity with respect to [ATP] (10-14). The data for bovine heart F$_1$, indicated three apparent $K_{ATP}^*$ values of the order of $10^{-6}$, $2 \times 10^{-4}$ and $10^{-3}$ M, of which the latter $K_{III}$ disappeared when F$_1$ was activated by assay in the presence of $\geq 10$ mM sodium bicarbonate (13). ATP hydrolysis by F$_1$ was also shown to display positive catalytic cooperativity in the sense that ATP binding to a second catalytic site increased turnover at the first site by 10$_{5}$-fold (turnover number $= 600$ s$^{-1}$) for the bovine heart F$_1$ (15; see also Ref. 16 for the Escherichia coli F$_1$). Furthermore, it has been shown that F$_1$ contains at least 6 nucleotide binding sites, of which 3 are considered to be catalytic and capable of binding various purine and pyrimidine nucleoside di- and triphosphates, and 3 noncatalytic and specific for adenine nucleotides (17, 18, see also Ref. 19).

The above important findings regarding the mechanism of ATP hydrolysis by F$_1$ persuaded us 4 years ago to embark on a detailed study of the kinetics of ATP synthesis by mitochondria. Prerequisite for this work was availability of a highly active, well-coupled, stable, and reproducible enzyme source. Minor modifications of the procedure of Hansen and Smith (20) yielded the desired SMP preparations, which were capable of ATP synthesis at a rate of 2000-3000 nmol (min. mg of protein)$^{-1}$ at 30°C, and retention of this activity for months at $-70$ °C (21).

The first phase of our studies showed that the kinetics of ATP synthesis were modulated by the coupled rate of respiration in the following manner. At low respiration rates and low steady-state $\Delta\psi$, the kinetics of ATP synthesis in an ADP concentration range of 1-1200 $\mu$M were monophasic, yielding linear Eadie-Hofstee plots ($v/[S]$ versus $v$) with apparent $K_{ADP}^{*} = 2-4$ $\mu$M and $V_{max} = 200$ nmol of ATP (min. mg of protein)$^{-1}$. At high respiration rates and high steady-state $\Delta\psi$, the Eadie-Hofstee plots were also essentially linear in the above ADP concentration range, with apparent $K_{ADP}^{**} = 120-$ 160 $\mu$M and $V_{max} = 11,000$ nmol of ATP (min. mg of protein)$^{-1}$. At intermediate respiration rates, the Eadie-Hofstee plots...
were curvilinear and analyzable in terms of a minimum of 3 $K_{\text{m}}^{\text{ADP}}$ values, namely, the low and the high values indicated above plus an intermediate $K_{\text{m}}^{\text{ADP}}$. The extreme $K_{\text{m}}^{\text{ADP}}$ values of 2–4 and 120–160 $\mu$M appeared to be fixed. However, the intermediate $K_{\text{m}}^{\text{ADP}}$ required for the best fit of the kinetic data seemed merely to indicate that the transition from the low $K_{\text{m}}^{\text{ADP}}$ to the high $K_{\text{m}}^{\text{ADP}}$ is fluid rather than abrupt (21, 22). Comparable results were obtained with $\Phi$, as the variable substrate (21), and fragmentary evidence suggests that similar $K_{\text{m}}$ changes occur as a function of light intensity in photophosphorylation by chloroplasts (23, 24). As shown by the data given above, the transition from the low to the high respiratory rate involved a 50-fold increase in both $V_{\text{max}}$ and $K_{\text{m}}^{\text{ADP}}$, thus indicating that with a constant $V_{\text{max}}/K_{\text{m}}$ the increase in apparent $K_{\text{m}}^{\text{ADP}}$ is mainly a consequence of $k_{\text{cat}}$ increase.

Fractional inactivation of the $F_0$-$F_1$ complex of SMP at either $F_0$ or $F_1$ was shown to result in (a) increase in steady-state $\Delta\psi$ (22, 25), (b) conversion of the kinetics of ATP synthesis to the high $K_{\text{m}}$ mode (21), and (c) increase in the turnover rate of the remaining, active $F_0$-$F_1$ complexes for ATP synthesis (26). Significantly, plots of the reciprocal of the rate of ATP synthesis per mol of active $F_0$-$F_1$ on the ordinate versus percent active $F_0$-$F_1$ on the abscissa were linear with either NaNAD or succinate as respiratory substrate, and the two lines intersected at the ordinate. The ordinate intercept indicated that the synthetic turnover number of $F_0$-$F_1$ is 440 s$^{-1}$ at 30 °C (26), which is essentially the same as the hydrolytic turnover number for SMP bound $F_1$, (400 520 s$^{-1}$) and the highest values reported for chloroplast photosynthesis (400–420 s$^{-1}$) (26, 27). The finding that upon phosphorylation before rapid NTP synthesis ensues.

The studies reported in this paper will show that at [ADP] < 1 $\mu$M and [GDP] and [IDP] below their respective $K_{\text{m}}$ values, the Eadie-Hofstee plots of the kinetics of NTP synthesis by SMP display positive cooperativity, and that there are two stages of high affinity NDF binding in oxidative phosphorylation before rapid NTP synthesis ensues.

**MATERIALS AND METHODS**

**Chemicals**

Unlabeled nucleotides were obtained from Pharmacia LKB Bio-technology Inc. Luciferin and luciferase were from Boehringer Mannheim. Carboxyatractyloside was from Sigma. Carrier-free [32P]P and [14C]ADP were from ICN. [PH]ADP and [3H]GDP were from Amer- sham Corp. The sources of all other chemicals were as indicated previously (13, 28).

**Preparation of SMP**

SMP were prepared from bovine heart mitochondria as described previously (21, 29). In the standard preparation, heavy layer bovine heart mitochondria are suspended in buffered sucrose solution containing 1.5 mM ATP before freezing at −70 °C as well as before sonication. This type of SMP will be referred to as A-SMP. Two other types of SMP were also prepared, one in which ATP was replaced with 10 mM GTP and another in which the added nucleoside triphosphate was 10 mM ITP. These preparations will be referred to as G-SMP and L-SMP, respectively. Except for the nucleotide changes indicated, other details were the same as those for the preparation of A-SMP. For nucleotide binding studies, certain preparations of G-SMP were made as follows.

Carboxyatractyloside Treatment—Mitochondria were incubated on ice for 10 min with 2 $\mu$mol of carboxyatractyloside plus 0.1 $\mu$mol of ATP/g of protein in order to arrest the adenine nucleotide carriers in the C-conformation (see also Ref. 30). Then, 10 mM GTP was added before freezing. The remainder of the procedure was the same as that indicated above for the preparation of G-SMP.

**KCl Wash**—This was done essentially according to the procedure described by Penefsky (31). The SMP were suspended at 1 mg/ml in a buffer containing 0.25 M sucrose, 10 mM Tris-SO4, pH 8.0, 2 mM EDTA, and 150 mM KCl, incubated at 30 °C for 20 min, then centrifuged at 50,000 rpm for 25 min in a Ti-60 rotor of the Beckman Model L-8 70M ultracentrifuge. The pellet was suspended in a buffer containing 0.25 M sucrose, 50 mM Tris-SO4, pH 8.0, 2 mM EDTA, 50 mM glucose, 5 mM MgCl2, 20 mM potassium phosphate containing 5–15 106 cpn of $^{3}P$, 70 pg of hexokinase/mg, 90 μg of SMP/ml, nucleotide at the concentrations indicated, and 0.5 mM NaNAD or 6.7 mM potassium succinate as the respiratory substrate. Regardless of the respiratory substrate and the nucleotide concentration employed, 32P esterification was linear with the time within the reaction time employed (4 min unless otherwise stated). ATPase activity was determined according to Pullman (33).

**Activity Assays**

Oxidative phosphorylation activity was measured at 30 °C essentially as reported previously (26). The assay mixtures contained 0.15 M sucrose, 10 mM Tris-SO4, pH 8.0, 2 mM EDTA, 50 mM glucose, 5 mM MgCl2, 20 mM potassium phosphate containing 5–15 106 cpn of $^{3}P$, 70 pg of hexokinase/mg, 90 μg of SMP/ml, nucleotide at the concentrations indicated, and 0.5 mM NaNAD or 6.7 mM potassium succinate as the respiratory substrate. Regardless of the respiratory substrate and the nucleotide concentration employed, 32P esterification was linear with the time within the reaction time employed (4 min unless otherwise stated). ATPase activity was measured according to Pullman (33).

**Estimation of Bound ATP and ADP in SMP Preparations**

Nucleotides were extracted from SMP as follows. SMP at 3 mg/ml in 0.25 M sucrose containing 50 mM Tris acetate, pH 7.5, were mixed with 0.67 volume of 1.25 M HClO4. After 5 min on ice, the mixture was centrifuged at top speed in a clinical centrifuge at 4 °C. The supernatant was removed and neutralized by addition of 0.09 volume of 6 N KOH, then centrifuged as before. The supernatant was assayed for ATP and ADP as described below. The amount of bound nucleotide was determined also in the F, fraction extracted from the SMP preparations. SMP at 5 mg/ml in 0.25 M sucrose containing 10 mM Tris-SO4, pH 7.5, were mixed with 0.5 volume of chloroform, vortexted for 10 s at room temperature, and centrifuged for 2 min in an IEC Clinical Centrifuge rotor No. 809 at the speed setting of 5. The supernatant was removed and centrifuged at room temperature in a Spinco Model L Ultracentrifuge No. 40 rotor for 30 min at 35,000 rpm. The supernatant from this second spin (F, fraction) contained 0.2–0.24 mg of protein/ml, and showed an ATPase activity of about 8 units/mg of protein. Acid extraction of nucleotides from the F, fraction was performed as described for SMP.

The amounts of ATP and ADP in the acid extracts were determined by the luciferin/luciferase method (34). The assay mixture at pH 7.5 contained, in a final volume of 1.5 ml, 50 mM Tris-SO4, 5 mM MgSO4, 0.5 mM EDTA, 1 mM phosphoenolpyruvate, 0.1 mM luciferin, and 7.5 μg of luciferase. To this mixture was added an aliquot (10–20 μl) of the extract, and light emission was continuously recorded in an SLM 8000 photoncounting spectrophotofluorometer. The extent of the emitted light, which is proportional to the ATP concentration of the sample, was stable during the course of the experiment (typically 7–8 min). To obtain the ATP concentration, 4 μl of pyruvate kinase (5 units/ml) were subsequently added to the mixture, which resulted after 3–4 min in a higher level of light emission. Finally, a known amount of ATP (10–20 pmol) was added as an internal standard. Because pyruvate kinase caused partial quenching, the degree of quenching was estimated in separate experiments by measuring light emission from ATP standards in the presence and absence of pyruvate kinase. Summarized in Table I are data regarding the ATP and ADP contents of A-SMP, G-SMP, I-SMP, and the $F_1$-rich fractions extracted therefrom.

**High Affinity Binding Measurement of Radiolabeled ADP and GDP**

In a final volume of 0.12 ml, G-SMP at 0.7–1.0 mg/ml in 0.25 M sucrose containing 5 mM MgCl2 and 50 mM Tris acetate, pH 7.5, were determined by the method of Lowry et al. (32).

2 M. Klingenberg, personal communication.
incubated at 30 °C with [³H]ADP (1.24 Ci/mmol) or [¹⁴C]ADP (53 mCi/mmol) for 30 s, or with [³H]GDP (0.47 Ci/mmol) for 3 min at the concentrations indicated in the appropriate figure legends. The SMP samples (100 μl) were then passed through 1-ml Sephadex centrifuge columns (G-50 equilibrated with the above medium). An aliquot of the eluate (70 μl) was mixed with 5 ml of Betain Blend and its radioactivity measured by a Beckman LS 1801 Scintillation Counter. The remainder of each eluate was used for determination of its protein concentration by the method of Lowry et al. (32). It was ascertained that under the conditions employed nonspecific leakage of radiolabeled nucleotide was negligible. For measurement of the ATP-nonchaseable fraction of bound [³H]ADP, MgATP was added at a final concentration of 2 mM after the initial 30-s incubation with [³H]ADP. Then, the mixture was further incubated for 1 min at 30 °C before applying it to a Sephadex centrifuge column.

RESULTS

Positive Cooperativity in GTP Synthesis—Shown in Fig. 1 are Eadie-Hofstee (v/[S] versus v) plots of the kinetics of GTP synthesis with GDP as the variable substrate and NADH (squares) or succinate (circles) as the energy source. Both plots are clearly convex, indicating positive cooperativity with respect to GDP. The data were analyzed with the assumption that at least 2 GDP molecules must bind before rapid GTP synthesis can be measured. This is shown in Equation 1 where E, S, and P are enzyme, substrate, and product, respectively, Kₐ is the dissociation constant for binding of the first substrate molecule, and Kₐ the dissociation constant for binding of the second substrate molecule (for definitions of K and see below). The rate equation used for calculation of Kₐ, Kₐ, and Vₘₐₓ is shown in Equation 2. It is assumed that ES does not produce product at a rapid enough rate to influence the measured rate of product formation by SES; therefore, the slow rate of product formation in the upward phase of Fig. 1 is due to low levels of SES.

\[
E + S \rightleftharpoons ES \rightleftharpoons E + P
\]

\[
v = \frac{V_{\text{max}}[S]}{K_{\text{m}}S + K_{\text{m}}[S]}
\]

As before (21, 22), values for the constants Kₐ, Kₐ, and Vₘₐₓ were derived from computer-assisted curve fitting. The dots in Fig. 1 show the fit of the assigned constants in Equation 2 to the experimental points. These assigned constants were: Kₐ = 99 μM, Kₐ = 972 μM, and Vₘₐₓ = 509 nmol of GTP (min·mg of protein⁻¹) with NADH as the respiratory substrate, and Kₐ = 53 μM, Kₐ = 490 μM, and Vₘₐₓ = 323 nmol of GTP (min·mg of protein⁻¹) with succinate as the respiratory substrate. As will be seen below, there exists a still higher affinity substrate-binding phase, which is not discernible from Eadie-Hofstee plots. Therefore, we shall refer to these binding sites in order of their decreasing substrate affinity as sites I, II, and III, and to the associated constants as Kᵢ, Kᵢ, and Kᵢ. Furthermore, since at [NDP] ≫ Kᵢ, all the free enzyme has been converted to ES and Kᵢ ≈ Kᵢ (see Equation 1), we shall refer interchangeably to the Kᵢ values determined here and the apparent Kᵢ values reported previously (21, 22).

Positive Cooperativity in ATP Synthesis—The demonstration of positive cooperativity in GTP synthesis made it necessary to re-examine the kinetics of ATP synthesis, because in our previous studies with variable ADP at 1–1200 μM there was no hint of positive cooperativity (21, 22). Examination of the kinetics of ATP synthesis at 0.1–800 μM ADP again failed to reveal positive cooperativity, as seen in the top trace of Fig. 3A. However, the problem was correctly diagnosed as being due to the presence of SMP-bound ATP, which direct analysis showed to be 3.3 nmol of ADP + ATP per mg of protein (Table I). Thus, during preparation of SMP, the added ATP was replaced with GTP, as described under “Materials and Methods.” The rationale was that GTP would chase off the exchangeable, bound ADP/ATP, then itself binding much less tightly would come off upon dilution of the SMP, leaving these sites vacant. Direct analysis of the SMP

![Eadie-Hofstee plots of the kinetics of GTP synthesis showing positive cooperativity with respect to GDP](image)

**Fig. 1.** Eadie-Hofstee plots of the kinetics of GTP synthesis showing positive cooperativity with respect to [GDP]. SMP were prepared in the presence of 10 mM GTP (G-SMP). Oxidative phosphorylation activity was measured as described under “Materials and Methods” with GDP as the variable substrate at 20–3000 μM. The respiratory substrate was 0.5 mM NADH (●) or 6.7 mM succinate (○). The data were analyzed according to Equation 2, and the dots represent the curves calculated from the Kᵢ, Kᵢ, and Vₘₐₓ values stated in the text. The Kᵢ, Kᵢ, and Vₘₐₓ values with IDP as the variable substrate, NADH as the energy source, and G-SMP as the catalyst were, respectively, 55 μM, 746 μM, and 522 nmol of ITP (min·mg protein⁻¹).
by the data of Fig. 2, this positive cooperativity phase at low phorylation assay pH was changed from pH 7.5 to 8.5 (Fig. 3A, open circles). Analysis of the lower traces of Fig. 3, A and B (closed circles), in terms of $K_{II}$, $K_{III}$, and $V_{max}$ is shown in Table II. It is seen that the $K_{II}$ values are essentially the same as the apparent $K_{III}$ values reported previously (21, 22), and that $K_{II}$ for the positive cooperativity phase was about 0.15 $\mu$m at pH 7.5, and 0.45 $\mu$m at pH 8.5.

High Affinity Binding of $[^{3}H]GDP$ and $[^{3}H]ADP$. The possibility of nucleotide binding sites with greater affinity than site II for ADP and GDP was investigated by direct binding experiments. Shown in Fig. 4 is a Scatchard plot of the binding of $[^{3}H]GDP$ to G-SMP at 30 °C. The $K_{d}$ determined from the slope of the line was 8.1 $\mu$m, which together with the data of Fig. 1, indicated that at pH 7.5 GDP binds at two sites with $K_{III}$ 8 $\times$ 10$^{-6}$ M and $K_{III}$ = 50–100 $\times$ 10$^{-6}$ M before rapid product formation takes place at higher GDP concentrations. The abscissa intercept of Fig. 4 indicated that 0.27 nmol of GDP was bound at site I/mg of G-SMP.

This value is close to the concentration of F$_1$ in A-SMP (0.4–0.45 nmol/mg of protein) as was determined immunologically (26). Whether G-SMP contain as much or less F$_1$ has not been determined, but we suspect that a fraction of the bound ADP + ATP of G-SMP (1.0 nmol/mg of protein) is at

![Fig. 3. Eadie-Hofstee plots of the kinetics of ATP synthesis by A-SMP and G-SMP at pH 7.5 and 8.5.](image)

![Fig. 4. Scatchard plot of the high affinity binding of $[^{3}H]GDP$ to G-SMP.](image)
site I (see below). It should be pointed out that the experiment of Fig. 4 was carried out with G-SMP prepared from carboxyatractyloside-treated mitochondria in order to arrest the adenine nucleotide carrier sites in the C-conformation and thereby exclude these sites from nucleotide binding in G-SMP. However, experiments with G-SMP not treated with carboxyatractyloside gave the same total number of high affinity GDP (and ADP, see below) binding sites as shown in Fig. 4. The noninterference of the carrier sites in these experiments was expected, of course, because all the binding experiments were conducted in the presence of 2 mM MgCl₂ and the Mg chelates of the nucleotides are considered not to bind to the adenine nucleotide carrier (35).

The finding of a binding site for GDP with higher affinity than site II ($K_I^{DP} = 50 - 100 \times 10^{-6}$ M) suggested that for ADP also there might be a binding site with an affinity greater than that estimated from the positive cooperativity phases of the kinetic data of Fig. 3, A and B. Results of binding experiments with [³H]ADP confirmed this expectation. Experiments with G-SMP yielded biphasic Scatchard plots (Fig. 5) with a lower affinity phase $K_d = 4 \times 10^{-7}$ M, which agreed with the $K_{II}^{DP}$ values derived from the positive cooperativity phases of Fig. 3, A and B (see Table II). However, the abscissa intercept indicated a capacity of only 0.25 nmol/mg of protein for sites I and II combined (Fig. 5), which suggested that G-SMP still contained bound adenine nucleotides at these sites. Thus, the adenine nucleotide content of the particles was further diminished by treatment with 150 mM KCl at pH 8.0, as described under "Materials and Methods." The KCl-treated G-SMP showed upon analysis a total adenine nucleotide content of 1.1 nmol (1.0 nmol of ATP, 0.1 nmol of ADP) per mg of protein, and considerable loss of oxidative phosphorylation activity. However, the Scatchard plot of data for [³H]ADP binding (Fig. 6) now appeared reasonable enough for estimation of $K_I^{DP}$ and $K_{II}^{DP}$. These values, as calculated from Fig. 6, were $K_I^{DP} = 0.7 \times 10^{-7}$ M, site I capacity $= 0.15$ nmol/mg of protein; $K_{II}^{DP} = 3.6 \times 10^{-7}$ M, site II capacity $= 0.45$ nmol/mg of protein. Once again, the $K_{II}^{DP}$ value agreed with the data of Table II, and site II capacity agreed with the $F_1$ content of SMP as stated earlier. We feel, however, that the data for site I are still rough estimates, and should be regarded not in terms of their magnitude, but only as an indication of the existence of such a high affinity ADP binding site in SMP. This conclusion is strengthened by the fact that bovine heart SMP are capable of tight binding of ATP, with $K_s = 10^{-2}$ M⁻¹ as determined from the association and dissociation rates of [γ-³²P]ATP (36).

Our final point deserves consideration with regard to nucleotide binding sites I and II, and that whether these sites are among the so called "exchangeable" sites. The question does not pertain to site III, because it is catalytic, therefore, necessarily an exchangeable site. That site II is an exchangeable site is indicated not only by the ATP chase data of Fig. 6 (closed circles), but also by the fact that addition of low levels of trinitrophenyl-ADP to a GDP phosphorylation assay mixture increased both $K_I^{DP}$ and $K_{II}^{DP}$, while $V_{GTP}^{max}$ remained unchanged (data not shown). Data demonstrating that site I is also an exchangeable site are given in Fig. 7, A and B, for ADP and GDP, respectively. It is seen that in each case 90% of the bound [³H]ADP or [³H]GDP of G-SMP was chased out upon addition of 2 mM MgATP (or MgADP) to the incubation mixture.

**FIG. 6. Scatchard plot of the high affinity binding of [³H]ADP to KCl-washed G-SMP.** KCl-washed G-SMP (see "Materials and Methods") at 0.75 mg/ml were incubated with 0.011-5 μM [³H]ADP at 30 °C for 30 s. Total [³H]ADP bound (O) was measured as described under "Materials and Methods." The data were analyzed in terms of two straight lines (shown in dots), each representing a single binding site. The curve through the experimental points is the calculated sum of the two lines. In O, the particles were incubated with 2 mM MgATP at 30 °C for 1 min after the initial 30-s incubation with 0.04-2.2 μM [³H]ADP. Then, the amount of bound [³H]ADP, which represents the ATP-nonchaseable fraction, was determined as described. The units for the abscissa and the ordinate are the same as described in the legend to Fig. 4.

**DISCUSSION**

The results presented above can be summarized in the two schemes in Fig. 8 for ATP and GTP synthesis. The $K_{II}^{P}$ (apparent $K_{II}^{DP}$) and $k_{cat}$ values for ATP synthesis are from previous studies (21, 22, 26; see also Table II). These schemes show three steps of ADP or GDP binding, of which only the last step leads to rapid ATP or GTP synthesis. As expected, the $K_i$, $K_{II}$, and $K_{III}$ values for GDP are 2 to 3 orders of

**FIG. 5. Scatchard plot of the high affinity binding of [³H]ADP to G-SMP.** G-SMP at 0.72 mg/ml were incubated with 0.25-5 μM [³H]ADP at 30 °C for 30 s. The amount of bound [³H]ADP was estimated as described under "Materials and Methods." The units for the abscissa and the ordinate are the same as described in the legend to Fig. 4.
Coupled rate of respiration and the rate of ATP synthesis are increased. Thus, Fig. 8 gives the extreme values shown are for the low $K_\text{m}$ (low respiration rate, low steady-state $A_+^*$) and high $K_\text{m}$ (high respiration rate, high steady-state $A_+^*$) modes. For GTP synthesis, the $K_\text{m}$, $K_\text{cat}$, and $k_\text{cat}$ values shown are with NADH as the respiratory substrate.

we have indicated in Fig. 8 the values derived from Fig. 1 where NADH was the respiratory substrate. However, it is clear from the Fig. 1 plot in which succinate was the respiratory substrate that these values are smaller when the coupled rate of respiration is diminished. Furthermore, as seen in Fig. 1, $K_{\text{DP}}^*$ was also affected by the rate of energy production by the respiratory chain. The effect of energy on $K_\text{m}$ also needs to be investigated, which is technically rather difficult to accomplish in the type of binding experiments employed here, because high concentrations of respiring SMP will rapidly exhaust the medium oxygen leading to collapse of the membrane potential. However, literature data regarding the lowered affinity of SMP upon energization for unisite binding of ATP (37) may be taken as an indication that energy might affect $K_{\text{DP}}^*$ and $K_{\text{DP}}^*$ as well.

It is perhaps important to compare the results for NTP synthesis with those reported by others for NTP hydrolysis. As pointed out in the Introduction, unisite ATP binding is very tight ($K_\text{m} = 10^{-12}$ M) and hydrolysis very slow (turnover number = $4 \times 10^{-3}$ s$^{-1}$) (2). $K_\text{m}$ for unisite ADP binding to SMP has not been determined (36), but the value of $10^{-9}$ M given for isolated bovine heart F$\text{I}$ (15) is not too far from our rough estimate of $K_{\text{DP}}^* \leq 10^{-8}$ M. Therefore, it is possible that $K_\text{ATP}^*$ represents the dissociation constant for unisite ADP binding at F$\text{I}$ catalytic sites. If so, it is also possible that there is a very slow unisite ATP synthesis, which, of course, would not be detectable under our assay conditions. To our knowledge, there is no definitive literature evidence regarding bisite ATP hydrolysis catalyzed by unmodified F$\text{I}$. What is known, however, is that inhibitory modification of one $\beta$ subunit/F$\text{I}$ (e.g. by dicyclohexylcarbodiimide, 4 chloro 7 nitrobenzoazan, or the ATPase inhibitor protein) diminishes the rate of ATP hydrolysis by >90% (13, 38-41). This may be an indication of the bisite turnover capability of F$\text{I}$, i.e. between the slow unisite rate and <10% of the trisite rate. To simplify calculations, we have assumed that the contribution of site II turnover to the measured rate of NTP synthesis is negligible, and that NTP synthesis in the positive cooperativity phase of Figs. 1 and 3 is the result of low levels of site III occupation by NDP. However, by comparison with data for the hydrolytic activity of F$\text{I}$ modified at 1 $\beta$ subunit/mol, it is possible that the synthetic activity of the positive cooperativity phase of our data includes contributions from site II turnover. Another possibility is that sites I and II are regulatory sites with no catalytic capability, and that their occupation by substrate is essential for enzyme turnover.

With sites I, II, and III occupied by substrate, rapid NTP synthesis ensues, but, as demonstrated previously, the kinetics of NTP synthesis are rather complicated. By changing the coupled rate of respiration and steady-state $\Delta \psi$, one observes a 50-fold change in both $V_\text{max}$ for ATP synthesis and apparent $K_{\text{ATP}}^*$, $K_{\text{DP}}^*$ also changes in the same direction. We have also demonstrated in other studies that the synthetic turnover rate of the F$\text{O}$-F$\text{I}$ complexes can be increased considerably by increasing steady-state $\Delta \psi$, and that this turnover rate reaches a maximum of 440 s$^{-1}$ with either NADH or succinate as the respiratory substrate, indicating that the F$\text{O}$-F$\text{I}$ complexes are saturable with respect to energy (26). Thus, one can think of energy as a substrate of F$\text{O}$-F$\text{I}$ complexes during ATP synthesis, whose degree of saturation of the enzyme at F$\text{I}$ affects both $V_\text{max}$ and apparent $K_\text{m}$ for ADP and Pi at F$\text{I}$. As stated above, these kinetic changes occur at constant $V_\text{max}/K_\text{m}$, which is consistent with our previous results showing that, in the limited range of substrate concentrations then used, the overall kinetics of oxidative phosphorylation and ATP-driven reverse electron transfer were suggestive of a two-site rapid
equilibrium random ping-pong mechanism (42). However, it is important to recall that, except at the very extremes of the coupled rate of energy production and steady-state $\Delta\Psi$, the kinetics of ATP synthesis in the ADP concentration range of 1–1200 $\mu$M are curvilinear (see Fig. 3) and include contributions from the low $K_{\text{m}Dp}$ of 2–4 $\mu$M, the high $K_{\text{m}Dp}$ of 120–160 $\mu$M, as well as intermediate, variable $K_{\text{m}Dp}$. These complex kinetic features may be related to cooperative interactions between sites I, II, and III, and the manner in which these interactions are affected by energy saturation (degree of protonation?) of the enzyme.

We have in previous publications compared the synthetic turnover rates and the energy-dependent $K_{\text{m}}$ and $V_{\text{max}}$ changes of the processes of mitochondrial oxidative phosphorylation and chloroplast photophosphorylation to the extent that limited available information regarding the latter has allowed. It seems that positive cooperativity in oxidative phosphorylation is yet another feature that might have a parallel in chloroplast photophosphorylation. Thus, it has been shown that ADP and ATP partially inhibit the ferricyanide reductase activity of spinach chloroplasts when added in amounts stoichiometric to the processes of mitochondrial oxidative phosphorylation. It has been shown that rapid photophosphorylation also requires the binding of ADP at more than one site.

Acknowledgments—We thank Drs. Carla Hekman and Takao Yagi for helpful discussions, and C. Munoz for the preparation of mitochondria.

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