MgADP and Free P_i as the Substrates and the Mg^{2+} Requirement for Photophosphorylation†

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ABSTRACT: Previous studies have not provided definitive information about whether ADP or P_i or their complexes with Mg^{2+} serve as substrates for photophosphorylation and whether free Mg^{2+} or ADP is required. Results presented show MgADP, MgGDP, or MgUDP are substrates. At variable Mg^{2+} concentrations, observed velocities are determined by MgADP and not the free ADP concentration. The approximate K_m for MgADP with spinach chloroplasts is about 30 μM, for MgGDP 260 μM, and for MgUDP above 5 mM. The apparent K_m values for added ADP or Mg^{2+} are decreased to constant low values near 30 μM as the added Mg^{2+} or ADP concentrations, respectively, are increased to the millimolar range. With 100 μM added Mg^{2+}, near-maximal velocities can be obtained with excess ADP, but not with excess GDP or UDP. This is explainable by the apparent K_m values for MgGDP and MgUDP being well above 100 μM. High phosphorylation rates with excess of either Mg^{2+} or ADP present show that little or no (<2–3 μM) free Mg^{2+} or ADP is required. In addition, the results show that during rapid photophosphorylation, when one or more catalytic sites are always filled with nucleotide, free ADP does not combine and block the combination of MgADP to catalytic sites that become vacant. This is in contrast to the ability of free ADP to combine tightly with one catalytic site when all catalytic sites are empty. The apparent K_m for added ADP above a few micromolar concentration, and with excess Mg^{2+} present, results from binding of MgADP at a second catalytic site. In contrast to the behavior of ADP, the apparent K_m for P_i is independent of the Mg^{2+} concentration, showing that free P_i is the substrate. In addition, some deviations of photophosphorylation rates from simple Michaelis–Menten relationships are noted and discussed.

The light-activated ATPase of chloroplast thylakoid membranes and the separated CF_1 ATPase bind ADP tightly at a catalytic site in the absence of medium Mg^{2+}. When catalytic-site ADP is present, Mg^{2+} can bind at micromolar concentrations and cause the ATPase activity to be strongly inhibited [see Du and Boyer (1989) and Murataliev et al. (1991)]. This reflects Mg^{2+} binding at one or possibly more of the multiple high-affinity Mg^{2+} binding sites known to be present on the enzyme (Hiller & Carmeli, 1985).

The ability of one catalytic site of CF_1 to bind ADP strongly in the absence of free Mg^{2+} raises the possibility that free ADP might be the substrate for photophosphorylation. If so, any catalytic-site Mg^{2+} required for photophosphorylation could bind prior to or after the ADP or could be bound in a complex with P_i. In conventional photophosphorylation assays with 5–10 mM added Mg^{2+}, most of the medium ADP will be present as the MgADP, and if free ADP were a required substrate, the affinity for the enzyme would need to be high. If any catalytic-site Mg^{2+} required for photophosphorylation binds to the enzyme before ADP, or is furnished in a complex with P_i, the Mg^{2+} in a MgADP complex would need to depart as the nucleotide combines with the enzyme.

If catalytic sites participate in sequence as suggested for the binding change mechanism, then with ADP or MgADP above micromolar concentrations one catalytic site will always have tightly bound reactants present and a second or third catalytic site may not have sufficient affinity for free ADP to make it a likely substrate. This and other considerations were the impetus for us make the assessments reported in this paper.

If MgADP serves as the preferred substrate, rates observed as the ADP concentration is increased at variable fixed Mg^{2+} concentrations should be determined by the concentration of MgADP present and not the total concentration of ADP added. If any catalytic-site Mg^{2+} necessary for ATP synthesis is furnished by MgADP, and if free ADP can bind to the catalytic site with about equal or higher affinity than MgADP, then with limiting Mg^{2+} excess free ADP should be a strong inhibitor.

Adequate assessment of whether ADP or MgADP or both serve as substrates for photophosphorylation does not appear to have been made. Measurements of apparent K_m values for ADP have been made with excess Mg^{2+} present (Bennum & Avron, 1965; Vinkler, 1981; Franek & Strotmann, 1981; Bickel-Sandkotter & Strotmann, 1981; Aflalo & Shavit, 1983; Loehr et al., 1985; Stroop & Boyer, 1985; Davenport & McCarty, 1986; Quick & Mills, 1987; Bizouarn et al., 1991). In reviews on photophosphorylation, we have not found discussion whether MgADP or MgP_i are substrates or of Mg^{2+} requirement. The only pertinent reference we have located is a study by Komatsu and Murakami (1976) of the inhibition of photophosphorylation by ATP. From their results, they regarded it as probable that MgADP and MgP_i serve as substrates. We thus undertook the studies reported here on the substrates and the Mg^{2+} requirement for photophosphorylation. Our results show that MgADP and free P_i serve as substrates for photophosphorylation and that little or no free Mg^{2+} or free ADP is required for rapid ATP synthesis. In addition, some other interrelations of velocity, MgADP concentration, and energy input are considered.

Experimental Procedures

Isolation of Chloroplast Thylakoid Membranes. The chloroplast thylakoid membranes were isolated essentially as

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described by Du and Boyer (1990). Fresh market spinach leaves were processed in a Waring blender in a grinding solution which contained, at pH 8.0, 50 mM Tricine, 0.4 M sucrose, 0.2 M choline chloride, and 5 mM $MgCl_2$. The membranes were collected by centrifugation for 10 min at 3000*g* and washed once with grinding solution. The membranes were washed once more with a solution containing, at pH 8.0, 2 mM Tricine and 10 mM NaCl, collected by centrifugation for 5 min at 12000*g*, washed 3 times with a solution containing, at pH 8.0, 2 mM Tricine, 50 mM NaCl, and 1 mM $MgCl_2$, and then collected by centrifugation for 5 min at 3000*g*. Finally, the membranes were washed once again with grinding solution and resuspended in grinding solution to a chlorophyll concentration of 3–4 mg/mL. The thylakoid membranes were stored in liquid nitrogen.

Depletion of Medium Mg^{2+}. For measurement of the dependency of phosphorylation, removal of most of the medium Mg^{2+} was needed. The thylakoid membranes, isolated as described above, were washed 2 times with a washing buffer containing, at pH 8.0, 50 mM Tricine, 0.4 M sucrose, and 0.2 M choline chloride. Finally, the thylakoid membranes were resuspended in the washing buffer to a chlorophyll concentration of 2.5–3.0 mg/mL. The washed membranes, in the absence of added Mg^{2+} and with 1 mM ADP and 5 mM P_i, showed a very low photophosphorylation rate, corresponding to 2–3% of the maximum rate. They were used within 1–1.5 h after Mg^{2+} removal.

Photophosphorylation Assays. These were carried out at pH 8.0 and room temperature in a mixture containing 50 mM Tricine, 50 mM KCl, 20 mM sucrose, 40 units of hexokinase/mL, 30 mM glucose, and 50 μM phenazine methosulfate. K_2HPO_4, Mg^{2+}, and ADP were added as given under Results. A 20-μL aliquot of the thylakoid suspension was added to a 1-mL volume of reaction mix for each assay. After 10 s in the dark, the mixture was illuminated with stirring by a projector lamp providing heat-filtered white light, followed by 30 s in the light to assure steady-state conditions. A 20-μL trace of [^{32}P]P_i [(0.2–1) $\times$ 10^7 cpm] was added, and the mixture was illuminated for 20 s unless otherwise indicated. The light was turned off, and 1 mL of cold 1 N perchloric acid was added.

The [^{32}P]P_i was separated from the [^{32}P]glucose-6-P and any [^{32}P]ATP by an adaptation of the procedure of Sugino and Miyoshi (1964). For this, carrier ATP and G-6-P, to a final concentration of 1 mM, and 0.5 mL of 50 mM ammonium molybdate were added, followed by 0.5 mL of triethylamine (1 g/100 mL, pH 2–4) with mixing by vortexing. The samples were left in the ice bath for 5 min followed by centrifugation at 4 °C for 10 min. Then 10 μL of 1 M K_2HPO_4 was added to the supernatant solution with vortexing, followed by another centrifugation. A 1-mL aliquot of the supernatant solution was added to 10 mL of water for ^{32}P counting by Cerenkov radiation.

Estimation of ATP Synthase Concentration. The amount of active synthase was estimated by the amount of [^{3}H]ADP that could be tightly bound at a catalytic site and rapidly chased by unlabeled ADP (Zhou and Boyer, unpublished results). This measure of the amount of active enzyme present is preferred to estimations based on the amount of chlorophyll present and the approximate amount of synthase per milligram of chlorophyll.

Control of Light Intensity. The light intensity of a slide projector was controlled by a variable transformer. Maximum output voltage provided about 50 000 lx of heat-filtered light at a distance of 30 cm to the reaction vessel. This was used for most experiments. Moving the reaction vessel closer provided up to 100 000 lx of light. Reduction of the voltage by 70% gave a light intensity of about 12 300 lx at the reaction tube surface, and cut the photophosphorylation rate in half.

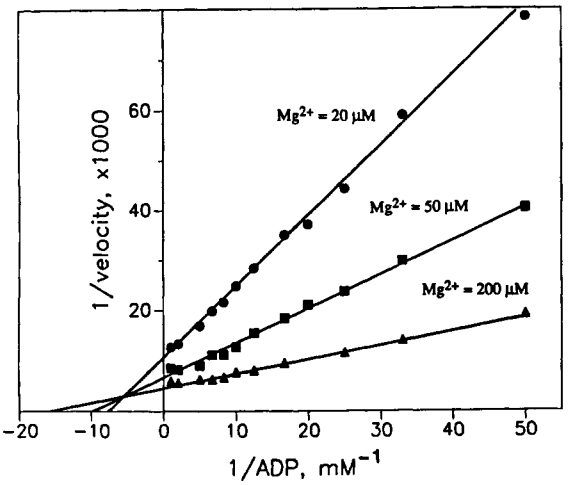

FIGURE 1: Photophosphorylation velocity at increasing added ADP concentration with fixed concentration of added Mg^{2+}. Ordinate, $1/v$ (ATP synthase^{-1} s^{-1}); abscissa, 1/[ADP] (mM). Spinach thylakoids were illuminated in the reaction mixture described under Experimental Procedures but containing 10 mM P_i and 20, 50, and 200 μM Mg^{2+}, respectively. The concentration of chlorophyll was 50 μg/mL.

RESULTS

Velocity Measurements with up to 200 μM Fixed Mg^{2+} Concentration and Increasing ADP Concentrations. Previous measurements of the dependence of the photophosphorylation rate on ADP concentration have been performed with 4–10 mM added Mg^{2+}, and thus nearly all the added ADP is present as MgADP. Apparent K_m values reported at or near pH 8 have ranged from 10 to over 100 μM on the basis of added ADP concentration (Bennun & Avron, 1965; Vinkler, 1981; Franek & Strotmann, 1981; Bickel-Sandkötter & Strotmann, 1981; Aflalo & Shavit, 1983; Loehr et al., 1985; Stroop & Boyer, 1985; Davenport & McCarty, 1986; Quick & Mills, 1987; Bizouarn et al., 1991).

To find if the amount of free Mg^{2+} present influenced the apparent K_m for ADP, measurements were made at pH 8.0 with variable [ADP] at three different concentrations of added Mg^{2+} and with excess P_i present. Plots of $1/v$ vs 1/[ADP] are shown in Figure 1 and gave apparent K_m values of 117, 80, and 56 μM on the basis of total ADP added at Mg^{2+} concentrations of 20, 50, and 200 μM, respectively. Although it seemed possible that the level of Mg^{2+} in the medium changed the affinity of the enzyme for ADP, it seemed more reasonable to explore if the differences in the apparent K_m would disappear if MgADP were considered to be the substrate.

The amounts of MgADP present under the conditions used for Figure 1 were calculated using a K_d value of 1.25×10^{-4} M for MgADP (Adolfsen & Moudrianakis, 1978). As shown in Figure 2, reciprocal plots for MgADP as a substrate were identical within experimental error for the three concentrations of total Mg^{2+} added. The estimated K_m value for MgADP from the plots is 30 μM.

If binding of Mg^{2+} to the catalytic site prior to ADP addition were required, normalization of the results based on the MgADP concentration would not be expected because the Mg^{2+} would be liganded to ADP and groups on the enzyme instead of just to ADP and water. If ADP should bind before Mg^{2+}, the affinity of the bound ADP for Mg^{2+} to form the

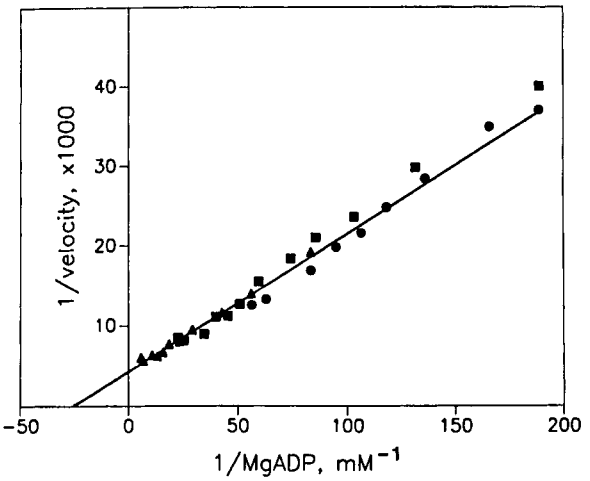

FIGURE 2: Velocity of photophosphorylation at increasing MgADP concentrations. Data are from Figure 1. Ordinate is the same as in Figure 1. Abscissa is 1/[MgADP].

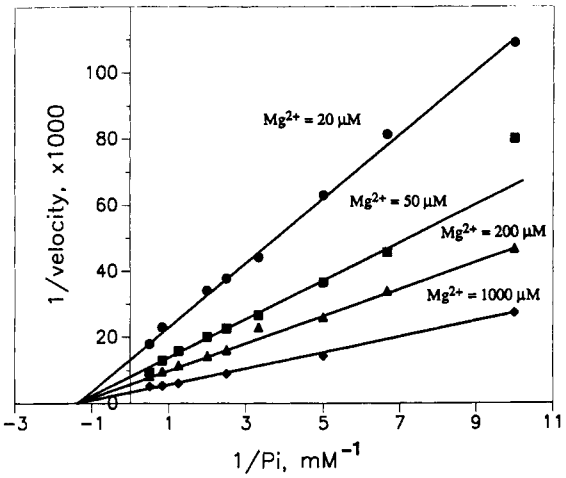

FIGURE 3: Photophosphorylation velocity at increasing added P_i concentration with fixed concentration of added Mg^{2+}. Ordinate, $1/v$ (ATP synthase^{-1} s^{-1}); abscissa, $1/[P_i]$ (mM). Spinach thylakoids were illuminated in the reaction mixture described under Experimental Procedures but containing 1 mM ADP and added Mg^{2+} at 20, 50, 200, or 1000 μM as indicated.

MgADP substrate apparently remains quite high. The results provide good evidence that the actual substrate for photophosphorylation is MgADP, a result confirmed by other trials reported later in this paper.

Velocity Measurements at Different Mg^{2+} Concentrations and Increasing P_i Concentrations. Measurements were made of the rate of photophosphorylation at pH 8.0 and with 1 mM added ADP. Results at different levels of added Mg^{2+} and with increasing P_i concentrations are shown in Figure 3 as plots of $1/v$ vs $1/[P_i]$. The rates observed with 20, 50, 200, or 1000 μM added Mg^{2+} gave the same apparent K_m value for P_i of about 700 μM. This is in sharp contrast to the results obtained with ADP, and are consistent with free P_i as the substrate that adds for photophosphorylation.

Effects of Excess ADP and Mg^{2+}. To find if excess ADP or Mg^{2+} were inhibitory and to assess whether free ADP or free Mg^{2+} were required for rapid photophosphorylation, rates were measured with increasing amounts of Mg^{2+} or ADP with different constant levels of added ADP or Mg^{2+}. Results are presented in Table I for rates of photophosphorylation with 20 or 1000 μM added Mg^{2+} and increasing [ADP] up to 2000 μM. With 20 μM added Mg^{2+}, free ADP up to 100 times the concentration of MgADP has little inhibitory effect. The

Table I: Photophosphorylation Rates with Increasing ADP or Mg^{2+} Concentrations at Fixed Concentrations of Added Mg^{2+} or ADP

[$MgCl_2$] added (μM)	[ADP] added (μM)	[Mg^{2+}], free (μM)	[ADP], free (μM)	[MgADP] (μM)	relative activity (%)
20	50	14.7	45	5.3	36
20	100	11.5	92	8.5	57
20	200	8.0	188	12.0	81
20	500	4.1	484	15.9	100
20	1000	2.3	982	17.7	95
20	2000	1.2	1981	18.8	87
1000	50	956	6	44	67
1000	100	912	12	88	94
1000	200	826	26	174	100
1000	500	588	88	412	99
1000	1000	297	297	703	95
1000	2000	102	1102	898	93
10	200	4	194	6	56
50	200	21	171	29	89
100	200	46	146	54	94
500	200	352	52	148	100
1000	200	826	26	174	97
5000	200	4805	5	195	87
10000	200	9803	2.5	198	75

participating catalytic sites show marked preference for MgADP. Reducing the free Mg^{2+} concentration below 2 μM by adding excess ADP likewise reduces the photophosphorylation rate only slightly, and what reduction is noted can logically be attributed to the excess ADP.

Also shown in Table I are photophosphorylation rates at a fixed added [ADP] and increasing added Mg^{2+} concentrations. With 200 μM added ADP, well above the apparent K_m for MgADP, addition of increasing [Mg^{2+}] first increased the photophosphorylation rate to a maximum rate at 500 μM added Mg^{2+}. Further increase in [Mg^{2+}] to the relatively high level of 10 mM gave up to 25% inhibition. The level of free ADP at the highest concentration of added Mg^{2+} was about 2.5 μM. The results show that there is no requirement for ADP greater than 2–4 μM for rapid photophosphorylation.

In trials such as above, the presence of the unusually high free Mg^{2+} or ADP concentrations did result in some decrease in reaction velocity. Possible explanations for this are considered under Discussion.

If MgADP is the substrate for photophosphorylation, and if the only complex formed from Mg^{2+} and ADP is MgADP, there should be reciprocal effects of variation of the concentration of either Mg^{2+} or ADP. For example, with 1 mM added ADP, increased concentrations of Mg^{2+} should give an apparent K_m for Mg^{2+} that is the same as the apparent K_m for ADP obtained with 1 mM added Mg^{2+} and increased concentrations of ADP. In addition, if all the added substrate partner is converted to MgADP by the excess of the other partner present, the apparent K_m value based on the total concentration of the variable substrate should be that found for MgADP. Experiments showed that the apparent K_m values for either ADP or Mg^{2+}, as estimated from the total added concentrations, decreased as expected when the fixed concentration of the other partner is increased. The value for the apparent K_m for Mg^{2+} reached 25 μM as the ADP concentration was increased, close to that found for MgADP (Figure 2). However, the apparent K_m for ADP approached 44 μM. This may reflect some inhibition by free ADP or other unrecognized factor(s). The important result is that apparent K_m values for MgADP were lowered to constant values not far from those obtained from velocity vs [MgADP] trials with little excess Mg^{2+} or ADP present.

Velocity Measurements at 5 mM Fixed Mg^{2+} Concentration and Variable ADP, GDP, and UDP Concentrations. GDP is

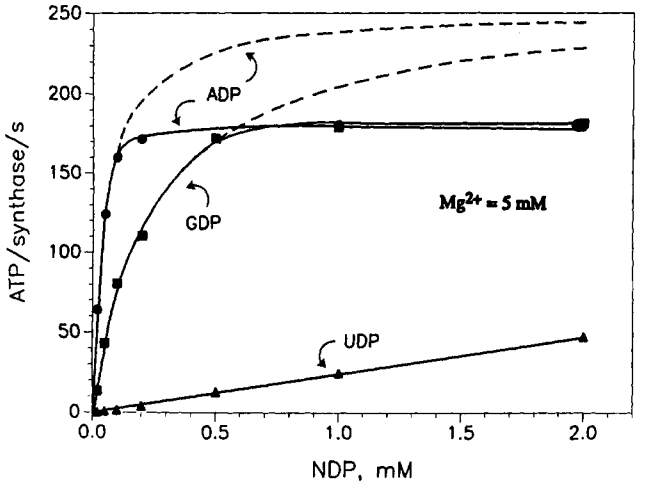

FIGURE 4: Photophosphorylation velocity at increasing concentration of added ADP, GDP, and UDP. Spinach thylakoids (chlorophyll, 20 μg/mL) were illuminated (5 s for ADP and GDP, 20 s for UDP, no hexokinase added) in the reaction mixture containing 50 mM Tricine, 50 mM KCl, 20 mM sucrose, 1 mM K_2HPO_4, 5 mM $MgCl_2$, and 50 μM phenazine methosulfate. The dashed line is a theoretical curve based on the simple Michaelis–Menten relationship.

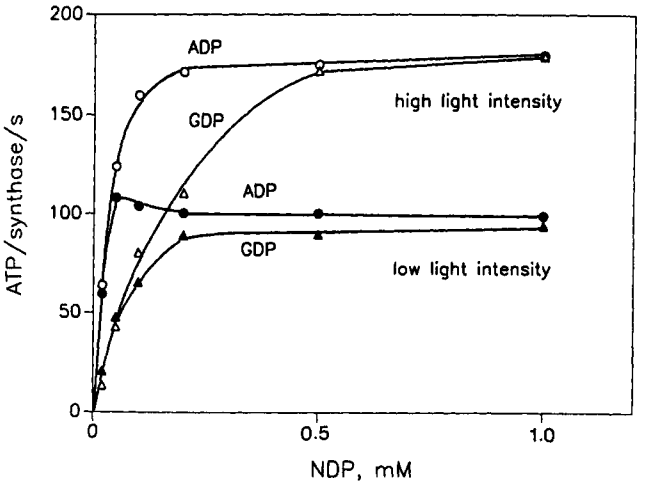

FIGURE 5: Effect of reduction of light intensity on the velocity with increasing added ADP and GDP concentration. Reaction mixture for photophosphorylation the same as for Figure 4. High light intensity was about 50 000 lx. Low light intensity was about 12 300 lx.

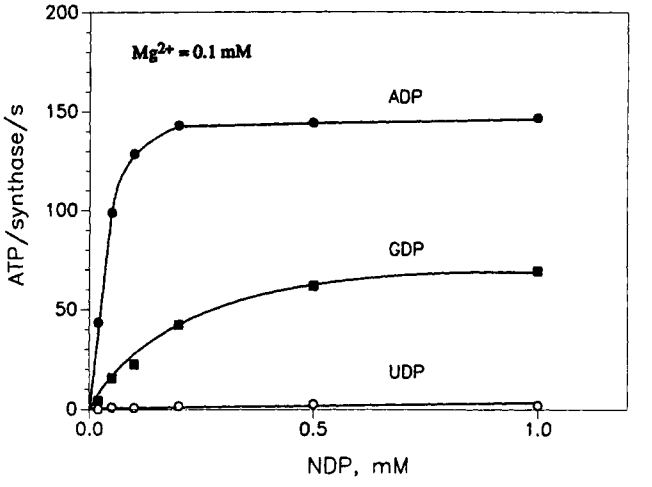

FIGURE 6: Photophosphorylation velocity at increasing concentrations of added ADP, GDP, and UDP in the presence of 0.1 mM Mg^{2+}. The reaction conditions were the same as for Figure 4.

phosphorylated rapidly (Bennum & Avron, 1965) and UDP poorly (Avron, 1960) by chloroplasts. They were tested to see if, as anticipated, MgGDP and MgUDP were substrates. In these tests, some anomaly was noted in photophosphorylation rates with relatively high ADP or GDP concentrations and with excess Mg^{2+} present. As shown in Figure 4, with an increase in [ADP] or [GDP], the increase in velocity leveled off somewhat abruptly when a rate of about 170 ATP synthase^{-1} s^{-1} was reached. Also shown in Figure 4 are theoretical curves based on the assumption that with both ADP and GDP the V_{max} was 250 ATP synthase^{-1} s^{-1}. They give an indication of how large the behavior differs from the rectangular hyperbola of a simple Michaelis–Menten relationship. This anomalous behavior obviously detracts from the accuracy of K_m estimations of 30 μM for ADP and 260 μM for GDP. The theoretical curves match the velocity points at lower substrate concentrations well, but determinations of the V_{max} and thus the K_m values would be improved if the anomaly did not occur.

The photophosphorylation rate response to UDP showed no indication of reaching a maximum rate with up to 2 mM UDP and excess Mg^{2+} (Figure 4). The K_m for UDP thus appears to be much higher than for ADP or GDP.

The explanation for the tendency of the velocity increase with increase in ADP or GDP concentrations to level off more rapidly than expected for Michaelis–Menten behavior is uncertain. With both ADP and GDP, slight inhibition by excess substrate occurs, but this alone is not sufficient to account for the results in Figure 4. It has been well recognized in the substrate–velocity studies cited earlier that as the photophosphorylation rate increases with increase in added ADP the ΔpH across the thylakoid membrane decreases. A reasonable possibility would seem to be that with the thylakoid preparations used the rate of energy supply to drive ATP formation becomes rate limiting as a velocity of 170–190 ATP synthase^{-1} s^{-1} is approached. Light intensity was not a limiting factor because with a doubling of light intensity the same dependency on added ADP was noted.

If the tendency to reach a plateau in velocity as shown with ADP and GDP is caused by a limitation of energy supply for ATP formation, then with a lowered light intensity, an apparent plateau should be reached with lower added ADP or GDP concentrations. This was found to be the case (Figure 5). The increase in velocity at the lower concentrations of ADP and GDP is close to the same as that at higher light intensity, but with less response and a rather abrupt tapering off at higher substrate concentrations. As substrate concentration is increased at low light intensity, the potential rate of utilization of protonmotive force soon exceeds the supply.

Velocity Measurements with Limiting Mg^{2+} Concentration and Increasing GDP and UDP Concentrations. If the nucleoside diphosphates combined with Mg^{2+} are the substrate for photophosphorylation, at limiting Mg^{2+} concentrations increases in the concentration of free nucleotides should not give the velocities attainable with excess Mg^{2+}. This is the case (Figure 6). With 100 μM Mg^{2+}, increases in added GDP and UDP gave observed maximum velocities much less than those observed with excess Mg^{2+} (Figure 4) and about as predicted on the basis of the expected concentrations of the MgNDP present calculated from the K_d for MgADP and the K_m values used for the theoretical curves in Figure 4.

Discussion

The results show that during rapid photophosphorylation MgADP and free P_i serve as substrates and that no or only very low (below about 2–4 μM) free ADP or Mg^{2+} is required. Demonstration that MgADP is a substrate is shown by the

photophosphorylation rates when [ADP] is varied at different added Mg^{2+} concentrations. The observed rates are determined by the [MgADP] and not the free ADP concentration (Figures 1 and 2). In contrast, the rate response to added P_i at different added Mg^{2+} concentrations is determined by the free P_i concentration (Figure 3).

That MgADP is the preferred substrate is also shown by the poor affinity of ADP for catalytic sites that are available during rapid photophosphorylation. Our study and earlier studies cited in the introduction show that a half-maximal rate of photophosphorylation is attained when 20–40 μM MgADP is present. The inability of high free ADP concentrations to inhibit the rate with 20 μM added Mg^{2+} (Table I) demonstrates the rather surprising low affinity of free ADP for available catalytic sites (Table I). With 1–2 mM ADP present, nearly all the added Mg^{2+} is present as MgADP. No more than 20 μM MgADP can be present, and the velocity is thus limited by MgADP concentration. Under these conditions, combination of free ADP at catalytic sites in preference to MgADP should sharply decrease the photophosphorylation rate. Such a decrease is not observed.

The preceding conclusion about ADP affinity is based on the elimination of an alternate explanation for the experimental results. This is that when ADP combines it creates a very high affinity binding site for Mg^{2+}; i.e., the affinity of enzyme·ADP for Mg^{2+} is markedly higher than that of free ADP. The K_d for the Mg^{2+} that combines with the CF_1·ADP may be as low as 4 μM (Murataliev et al., 1991). However, a ratio of free ADP to MgADP of over 100 to 1, leaving a free [Mg^{2+}] of about 1.2 μM (Table I), caused little velocity decrease. This means that any enzyme·ADP that formed would need to be nearly saturated with Mg^{2+} at a free Mg^{2+} concentration as low as 1 μM. Also, such behavior would mean that nearly maximal photophosphorylation rates should be attainable by ADP addition at relatively low Mg^{2+} concentrations. This is not as observed. These considerations support the interpretation that MgADP is markedly preferred to free ADP as the combining substrate.

That the Mg^{2+} required for photophosphorylation combines tightly with the enzyme and then nucleoside diphosphate substrates add is also made unlikely by the demonstration that 100 μM added Mg^{2+} suffices for near-maximal photophosphorylation rates attainable with an increase in the [ADP] but not with the GDP or UDP concentrations (Figure 6). If an enzyme with a tightly bound Mg^{2+} were the participating species, then increases in the GDP and UDP concentrations should give maximal rates without the requirement for more than 100 μM added Mg^{2+}. The higher Mg^{2+} concentration required to attain maximal photophosphorylation rates with GDP and UDP reflects the higher MgGDP or MgUDP concentrations required for maximal velocity.

The utilization of MgADP substantiates the suggestion of Komatsu and Murakami (1976), but our findings do not support their suggestion that MgP_i is a substrate. Comment is also appropriate that the lack of a requirement for medium Mg^{2+} does not mean that the chloroplast thylakoids do not have tightly bound Mg^{2+} remaining even though washed extensively. The presence of Mg^{2+} that is difficult to remove has been noted with the mitochondrial and *Escherichia coli* enzymes (Senior et al., 1980).

Comment is appropriate about the number of catalytic sites that might filled as the ADP concentration is increased. That more than one catalytic site is filled during rapid steady-state phosphorylation is supported by measurements of the rate with very low added ADP concentrations and with excess Mg^{2+} present. These revealed an apparent K_m value of about 0.6 μM and a very low V_{max} (Stroop & Boyer, 1985). A similar behavior has been shown with a bacterial synthase (Perez & Ferguson, 1990). Although this seems likely to reflect the addition to the first catalytic site, whether this low K_m reflects filling of a first or second catalytic site has not been established. The apparent K_m values for MgADP reported in this paper, and earlier measurements giving K_m values in the 20–40 μM range, are considered to reflect the binding to a second and/or possibly a third catalytic site. Any rate acceleration that might result from occupancy of a third catalytic site could be obscured by the weak inhibitions produced by excess Mg^{2+} or ADP.

The weak inhibition produced by excess ADP could result if two ADP molecules combined with portions of one active site, similar to the manner often suggested for inhibitions of single-site enzymes by excess substrate. We do not have reasonable suggestions to make as to the cause of the inhibition of photophosphorylation by excess Mg^{2+}.

The tendency for the increase in the photophosphorylation rate to level off rather abruptly as ADP or GDP concentrations increase (Figure 4) was somewhat unexpected because the previous investigations of velocity vs ADP relationships cited earlier did not report such behavior. However, measurements at ADP concentrations above about 100–200 μM were not reported in most instances. It seems likely that the behavior noted by us is not an isolated phenomenon. The best explanation we can suggest, as mentioned earlier, is that the provision of energy for binding changes at the catalytic site becomes rate-limiting. Our results show that this is not due to a limitation in light intensity. It could reflect limitation in production or maintenance of protonmotive force, or limitation in the utilization of protonmotive force by the F_o portion of the synthase.

Mention should perhaps be made that we are not aware of any reports that show whether ADP or MgADP serves as a substrate for oxidative phosphorylation. In view of the results reported here, it appears likely that MgADP is the substrate for all ATP synthases.

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Binding of Calcium by Calmodulin: Influence of the Calmodulin Binding Domain of the Plasma Membrane Calcium Pump†

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ABSTRACT: The interaction between calmodulin and synthetic peptides corresponding to the calmodulin binding domain of the plasma membrane Ca^{2+} pump has been studied by measuring Ca^{2+} binding to calmodulin. The largest peptide (C28W) corresponding to the complete 28 amino acid calmodulin binding domain enhanced the Ca^{2+} affinity of calmodulin by more than 100 times, implying that the binding of Ca^{2+} increased the affinity of calmodulin for the peptide by more than 10^8 times. Deletion of the 8 C-terminal residues from peptide C28W did not decrease the affinity of Ca^{2+} for the high-affinity sites of calmodulin, but it decreased that for the low-affinity sites. A larger deletion (13 residues) decreased the affinity of Ca^{2+} for the high-affinity sites as well. The data suggest that the middle portion of peptide C28W interacts with the C-terminal half of calmodulin. Addition of the peptides to a mixture of tryptic fragments corresponding to the N- and C-terminal halves of calmodulin produced a biphasic Ca^{2+} binding curve, and the effect of peptides was different from that on calmodulin. The result shows that one molecule of peptide C28W binds both calmodulin fragments. Interaction of the two domains of calmodulin through the central helix is necessary for the high-affinity binding of four Ca^{2+} molecules.

The affinity of Ca^{2+} for calmodulin is greatly enhanced by the addition of target enzymes and peptides. This is shown by Ca^{2+} binding studies with enzymes such as myosin light chain kinase and cyclic nucleotide phosphodiesterase (Olwin & Storm, 1985), caldesmon (Yazawa et al., 1987), or the calmodulin-binding peptides melittin (Maulet & Cox, 1983) and mastoparan (Yazawa et al., 1987). Melittin and mastoparan binds strongly to calmodulin in the presence of Ca^{2+} (Maulet & Cox, 1983; Malencik & Anderson, 1983). The studies using mastoparan indicate that communication between the N-terminal and C-terminal domains of calmodulin may play a role in enzyme activation (Yazawa et al., 1987, 1990). This is at variance with what is observed in the target-free, dumbbell-shaped calmodulin molecule (Babu et al., 1988).

The calmodulin binding domain of the plasma membrane Ca^{2+} pump has recently been identified as a 28 amino acid sequence located near the C-terminal portion of the molecule (James et al., 1988; Verma et al., 1988; Vorherr et al., 1990). It shares with other calmodulin-dependent proteins the propensity to form a basic amphiphilic helix (James et al., 1988). Synthetic peptides corresponding to the calmodulin binding domain of the pump have been shown to interact with calmodulin with K_ds in the nanomolar to subnanomolar range (Vorherr et al., 1990). In addition, the peptides inhibit strongly the activity of the Ca^{2+} pump (Enyedi et al., 1989).

In the work presented in this contribution, the interaction between calmodulin and the synthetic peptides corresponding to the calmodulin binding domain of the Ca^{2+} pump was studied by monitoring the binding of Ca^{2+} to calmodulin. A 28-residue peptide (C28W) was found to enhance very significantly the Ca^{2+} binding to calmodulin. The enhancement was also observed with a mixture of two calmodulin fragments, corresponding to the N-terminal and C-terminal halves of the molecule, respectively. However, the Ca^{2+} binding curve of the mixture of the two fragments and peptide C28W became biphasic due to the decreased Ca^{2+} binding to the N-terminal domain. The work underlines the importance of interdomain interaction through the central helix for calmodulin function.

Materials and Methods

Calmodulin was prepared from scallop testis as described previously (Minowa & Yagi, 1984). When it is compared to vertebrate calmodulin, the scallop protein has three conservative substitutions among 148 amino acid residues (Tyr99-Phe, Gln143-Thr, Ala147-Ser) (Toda et al., 1981). Other properties such as Ca^{2+} binding and enzyme activation profiles are the same as for vertebrate calmodulin. Tryptic fragments (F12 and F34) of calmodulin were prepared by digestion in the presence of 2 mM $CaCl_2$ and purified by DEAE-cellulose chromatography as described in a previous paper (Minowa & Yagi, 1984). Fragment F12 (acetyl-Ala1-Lys75) corresponds

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