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The Role of Mg^{2+} in the Hydrolytic Activity of the Isolated Chloroplast ATPase: Study by High-Performance Liquid Chromatography

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The influences of total magnesium ion concentration at different total ATP concentrations, and of total ATP concentration, for different total magnesium ion concentrations, on the enzymatic rate of the isolated chloroplast F_1 ATPase, have been followed by a chromatographic method consisting in the separation and determination of ADP. From the various series of curves, it is concluded that the experimental results (position of the maxima, K_m values) are better fitted by a mechanism involving the activation of the enzyme by magnesium ion and hydrolysis of free ATP, rather than by the classical mechanism, for which the enzyme hydrolyzes the MgATP complex and is inhibited by Mg^{2+}. Although the equations giving the reaction rate are similar in the two cases, the calculated values of K_m are widely different. The value obtained from the classical mechanism does not agree with K_D, the dissociation constant of the enzyme–substrate complex, measured by the Hummel and Dreyer method. Moreover, when the total ATP concentration tends toward the total magnesium ion concentration, the nucleotide binding to the enzyme tends toward zero, although it should be maximum if MgATP were the true substrate. Finally, the inhibitory effect of Na^+ is more easily explained as a competition between this ion and the activating Mg^{2+}, than by the classical mechanism.

KEY WORDS: Chloroplast ATPase; reaction rate; HPLC; Mg^{2+}.

INTRODUCTION

The enzymatic behavior of the proton F_0–F_1 ATPase found in bacterial, mitochondrial, and chloroplastic membranes has been intensively studied for more than thirty years. The enzyme can catalyze the hydrolysis or the synthesis of ATP:

$$ATP \rightleftharpoons ADP + Pi$$

For the hydrolytic reaction, there is a fair amount of agreement on the hypothesis that MgATP is the active substrate, free ATP and free Mg^{2+} being inhibitors,[1–8] as stated a long time ago by Adolfsen and Moudrianakis: "The activation is indirect, being the result of complexation of inhibitory free ATP. Free ATP is a noncompetitive inhibitor of MgATP hydrolysis. The inhibition by free Mg^{2+} is direct, being the result of Mg^{2+} binding to a specific inhibitory site on the enzyme, which is distinct and separate from the MgATP binding site".[2] The general agreement on this hypothesis should not conceal that another hypothesis could also account for the experimental results: the enzyme is activated by free Mg^{2+}, and ATP, not MgATP, is the true substrate. It will be shown in this paper that this last hypothesis can account for the effect of Mg^{2+} and for the inhibitory effect of Na^+ or K^+, which cannot be easily explained by competition, if Mg^{2+} is also an inhibitor. It would also account for the acceleration of the hydrolysis rate when the total ATP concentration is increased, the so-called cooperative effect, attributed to the filling of regulatory sites.

Reexamining the question, we felt that an

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essential experimental parameter, namely the reaction rate, could have been measured under non-optimal conditions, leading to a biased analysis. Three methods have been most widely used to determine the rate of ATP hydrolysis by the F_1 protein: (i) measurement of the phosphate released from hydrolysis of ATP, without separation from other products,[9] or after solvent extraction of complexed Pi;[10] (ii) determination of protons produced by hydrolysis of ATP, with a pH-stat apparatus[11] or a pH indicator;[12] (iii) measurement of the released ADP, by means of a complex enzymatic system, allowing ATP to be regenerated. The enzymatic system includes phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase, and NADH, the absorbance of which is followed as a function of time.[13] A drawback of this last method is that it makes use of phosphoenolpyruvate at millimolar concentrations, a condition which may decrease the concentration of free Mg^{2+} by formation of a complex between phosphoenolpyruvate and Mg^{2+}.

In a recent note,[14] we advocated an HPLC method, in which ADP is separated from ATP on an anion-exchange column and its concentration measured by its absorbance. This method is quite simple and is less susceptible to possible artefacts than previously used methods. As reported in this paper, we used it for a systematic study of the effect of the concentration of various entities (ATP, Mg^{2+}, Na^+) on the rate of ATP hydrolysis by the purified ϵ-depleted chloroplast F_1. It was previously shown that this rate is constant during several minutes and proportional to the enzyme concentration.[14] We conclude that the enzyme is activated by the Mg^{2+} ion and that ATP, not MgATP, is its true substrate.

MATERIALS AND METHODS

Coupling factor CF_1 was extracted from spinach chloroplasts by sucrose chloroform treatment,[15] then adsorbed on DEAE cellulose and eluted by 0.4 M NaCl. It was further purified by HPLC on a Protein Pak 5 PW Waters column, eluted with an ammonium sulfate gradient (0–1 M, in 20 mM Tris, pH 8). The ϵ-depleted CF_1 ($CF_1 - \epsilon$), used in this study as active ATPase, was obtained by HPLC under the same conditions from CF_1 after freeze thawing, adsorption on the column, and extensive washing (1 hour) with 20 mM Tris buffer, pH 8.[16] ATPase activity was measured at 37°C in 0.075 M Tris sulfate buffer, pH 8.5, $MgSO_4$ from 0.0062 to 10 mM, with ATP concentrations ranging between 0.025 and 10 mM.

The chromatographic apparatus (Waters) consisted of a M 510 pump, a U_6K injector, and a 490 E detector, set at 260 nm. Aliquots of the reaction mixture were directly injected into a TSK DEAE 2SW column (30 × 0.4 cm). The nucleotides were separated by isocratic elution with H_2KPO_4 0.1 M, NaCl 0.25 M, at a rate of 1.5 ml/min. With this procedure, the reaction was immediately stopped after injection by the separation of the enzyme from the substrate and by the pH drop to 4.3. ADP concentration was measured by the height of the peak at the column exit. It increased linearly with time of action of the enzyme in the reaction medium, for a period of several minutes to several hours, depending on the percentage of hydrolyzed ATP and of released ADP acting as inhibitor. A calibration curve for ADP was established under the same conditions. ATP and ADP concentrations of standards were calculated from the absorption at 260 nm, with a molar absorption coefficient of 15,400. They were corrected for the presence of impurities determined by HPLC: AMP and ATP (in ADP) and ADP (in ATP).

Protein dosages were performed with the Bio Rad protein assay using bovine serum albumin as a standard or by UV absorption spectroscopy assuming for $CF_1 - \epsilon$ an $E^{1\%}_{1\,cm}$ of 4.8 at 280 nm. Rates of ATP hydrolysis were thus evaluated as rates of ADP formation, expressed in μmol per min per mg of protein.

The Hummel and Dreyer method[17] was used to measure the dissociation constant K_D of the equilibrium between the ATPase and its substrate. It is based on the following protocol: a known quantity of enzyme is injected into a chromatographic column equilibrated with a constant concentration of ligand (here, ATP). The amount of ATP that binds is proportional to the enzyme quantity. It depends on the dissociation constant and on the ligand concentration. This ATP is withdrawn from the eluent and migrates with the enzyme, while a trough in the ATP concentration profile runs with the ligand rate. This negative peak is measured; it gives the number r of molecules of ligand bound per molecule of enzyme. According to the multiple equilibrium theory, $r/(s)$ is linear versus r, where (s) is the free ligand concentration. The slope of the straight line is $1/K_D$ and the extrapolation on the x axis gives the number n of sites (Scatchard plot[18]). The advantage of this method is that there is no dissociation of the complex during the

chromatographic separation, since the complex is always in equilibrium with a constant concentration of ligand. It thus permits one to measure the K_D of low-affinity complexes. We have previously applied this method to the F_1 ATPase, in order to measure K_D and n with ADP and ATP, using a gel filtration column[19] or an anion exchange column.[20]

ATP (disodium salt) and ADP (sodium salt) from Sigma were brought to pH 8.5 by NaOH. Tris(hydroxymethyl)amino methane (Trizma Base, Sigma) was brought to pH 8.5 by H_2SO_4.

RESULTS AND INTERPRETATIONS

Influence of Magnesium Ion Concentration

We measured the influence of total Mg^{2+} concentration on the rate of ATP hydrolysis for several concentrations of total ATP, the enzyme concentration being kept constant. As shown in Fig. 1, the enzymatic rate presents a maximum as a function of the total magnesium concentration. The position of this maximum varies with the total ATP concentration but does not correspond to a simple integer value of the ATP/Mg^{2+} ratio (Table I).

In order to interpret this behavior, we have to consider that ATP, like other nucleotides, binds divalent cations, in particular Mg^{2+}, according to the equilibrium

$$ATP^{2-} + Mg^{2+} \rightleftarrows MgATP$$

The dissociation constant K_{as} of this equilibrium

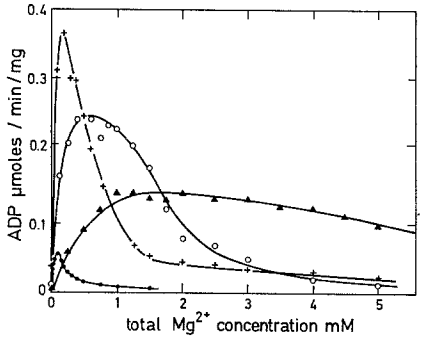

Fig. 1. Influence of the total Mg^{2+} concentration on the rate of ATP hydrolysis. Conditions described in the text. Final concentration of $CF_1 - \epsilon$: 59 µg/ml. (●) 0.284 mM ATP_t; (+) 0.914 mM ATP_t; (○) 2.71 mM ATP_t; (▲) 8.89 mM ATP_t.

Table I. Optimal Concentrations of Total Magnesium Ion for Different Concentrations of Total ATP

Total ATP concentration (mM)	0.284	0.914	2.71	8.89
Total Mg^{2+} concentration at the maximum of enzymatic rate (mM)	Between 0.05 and 0.075	0.18	0.5	1.6
Ratio Total Mg^{2+}/Total ATP at the maximum of the enzymatic rate	Between 0.18 and 0.26	0.20	0.185	0.18

has been measured by other groups using different methods such as spectrophotometry with a Mg^{2+} indicator acting by competition,[21,22] pH titration,[23,24] resin competition,[25] or ^{31}P NMR.[26] The values obtained ranged between 1 and 0.01 mM. The value most appropriate to our experimental conditions (ionic strength, temperature) and most recently obtained is 0.02 mM.[26] Assuming this dissociation constant value, the concentrations of free Mg^{2+} (a), of free substrate (s), and of MgATP complex (as), can be computed from the concentrations of total Mg^{2+} (a_t) and of total substrate (s_t). They are given by (see Appendix 1)

$$s^2 + s(a_t - s_t + K_{as}) - K_{as}(s_t) = 0 \qquad (1)$$

$$a^2 + a(s_t - a_t + K_{as}) - K_{as}(a_t) = 0 \qquad (2)$$

In the presence of the enzyme, these equations remain valid, since the quantities of substrate and of magnesium ion bound to the protein are negligible. In order to explain our experimental results, we propose the hypothesis that the enzyme is first activated by magnesium ion and that free ATP (i.e., not MgATP) is then hydrolyzed. Let us designate by E, A, S, EA, and EAS, the enzyme ($CF_1 - \epsilon$), the activator (Mg^{2+}), the substrate (ATP), and their complexes, respectively, that are involved in the equilibria:

$$E + A \rightleftarrows EA,$$

$$\text{with a dissociation constant } K_a = \frac{(e)(a)}{(ea)}$$

$$EA + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} EAS \xrightarrow{k_2} EAP \longrightarrow EA + P,$$

$$\text{with } K_m = \frac{k_2 + k_{-1}}{k_1}$$

The choice of this model, rather than the classical one, will be justified *a posteriori* in the discussion. With this hypothesis, the enzymatic rate is given by

(see Appendix 2)

$$v = \frac{k(e_t)}{1 + (1 + K_a/(a))(K_m/(s))} \tag{3}$$

This formula accounts for the variation of the position of the maximum of the enzymatic rate as a function of the total magnesium ion concentration and allows one to calculate K_a. Indeed, as long as the concentration of total magnesium ion is lower than that of total ATP (and in particular, in the region of the maximum of the enzymatic rate), it can be verified by Eqs. (1) and (2) that

$$(s) \approx (s_t) - (a_t)$$
$$(as) \approx (a_t)$$

Then

$$(a) = \frac{K_{as}(as)}{(s)} \approx K_{as} \cdot \frac{(a_t)}{(s_t) - (a_t)}$$

and (3) gives

$$v = \frac{k(e_t)}{1 + \left[1 + \frac{K_a((s_t) - (a_t))}{K_{as}(a_t)}\right] \cdot \frac{K_m}{(s_t) - (a_t)}}$$
$$= \frac{k(e_t)}{1 + \left[\frac{1}{(s_t) - (a_t)} + \frac{K_a}{K_{as}(a_t)}\right] K_m}$$

At the maximum, the derivative of the reciprocal is zero:

$$\frac{d(1/v)}{d(a_t)} = \frac{1}{k(e_t)}\left[\frac{K_m}{((s_t) - (a_t))^2} - \frac{K_a K_m}{K_{as}(a_t)^2}\right] = 0$$
$$\left[\frac{(a_t)}{(s_t) - (a_t)}\right]^2 = \frac{K_a}{K_{as}} \tag{4}$$

The ratio $[(a_t)/((s_t) - (a_t))]^2$ obtained from our measurements is nearly the same for concentrations of total ATP of 8.89, 2.71, and 0.914 mM: 0.048, 0.051, and 0.060, respectively. For a lower concentration (0.284 mM), the maximum has not been determined with accuracy (a_t between 0.05 and 0.075 mM), and the corresponding ratio is between 0.045 and 0.128. Assuming that $K_{as} = 0.02$ mM, the mean value for K_a would be 1.43 μM.

Equation (4) can also be written as

$$\frac{K_a}{K_{as}} = \left(\frac{(a_t)}{(s_t) - (a_t)}\right)^2 = \frac{(a)^2}{K_{as}^2} \quad \text{or} \quad K_a = \frac{(a)^2}{K_{as}}$$

The concentrations of free Mg^{2+} corresponding to the maximum of the enzymatic rate, calculated from Eq. (2) for the four values of total ATP used above, are, respectively, 4.39, 4.52, 4.90, and between 3.9 and 6.4 μM. The mean value of K_a, using this other method of calculation, is 1.38 μM.

Influence of ATP Concentration

The influence of ATP on the rate of hydrolysis was studied in greater detail using three different concentrations of total Mg^{2+}. The results are plotted in Fig. 2, which shows that the enzymatic rate is a complicated function of the total substrate concentration, depending also on the total magnesium concentration. At very low substrate concentration, the reaction rate varies linearly with that concentration (Fig. 3). Under these conditions most of the ATP is complexed with Mg^{2+}; the free substrate concentration is thus very low (in the micromolar range) and, as will be shown below, far smaller than the K_m value.

Equation (3) becomes

$$v = \frac{k(e_t)}{1 + (1 + K_a/(a)) \cdot (K_m/(s))}$$
$$\approx \frac{k(e_t)(s)}{(1 + K_a/(a))(K_m)} \approx \frac{k(e_t)(s)}{K_m}$$
$$(s) = \frac{K_{as}(as)}{(a)} \approx \frac{K_{as}(s_t)}{(a_t)}$$

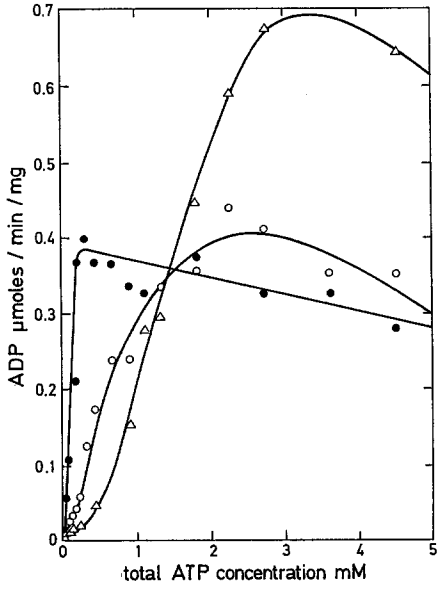

Fig. 2. Influence of the total ATP concentration on the rate of ATP hydrolysis. Conditions described in the text. Final concentration of $CF_1 - \epsilon$: 18 μg/ml. (●) 0.1 mM Mg_t^{2+}; (○) 0.33 mM Mg_t^{2+}; (△) 1 mM Mg_t^{2+}.

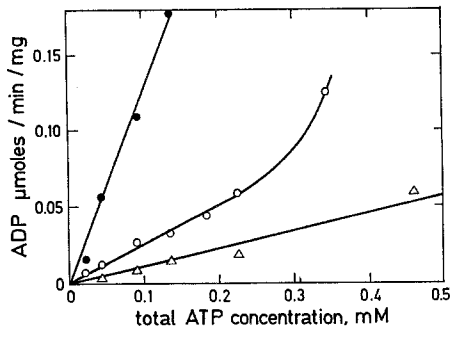

Fig. 3. Influence of total ATP concentration on the hydrolysis rate at low ATP concentrations. Same experiments as for Fig. 2, expanded to better show the results at low ATP concentration.

$$v \approx \frac{k(e_t)K_{as}}{K_m} \cdot \frac{(s_t)}{(a_t)}$$

$$\frac{v}{(s_t)} = \frac{k(e_t)K_{as}}{(a_t)K_m} \quad \text{or} \quad \frac{k}{K_m} = \frac{v}{(s_t)} \cdot \frac{(a_t)}{K_{as}(e_t)} \qquad (5)$$

The slopes of the straight lines (v/s_t) in the initial parts of the curves (Figs. 2 and 3) allow one to calculate the k/K_m ratio. The calculated values are 6.15, 3.92, and 4.12, giving a mean value of $4.73\,\text{ml}\,\text{min}^{-1}\text{mg}^{-1}$.

When the total substrate concentration exceeds the total magnesium concentration, the free substrate concentration [calculated from Eq. (1)] increases rapidly and linearly with the difference $(s_t) - (a_t)$, as can be seen in Fig. 4. This behavior is simply due to the high affinity of ATP for Mg^{2+} ($K_{as} = 0.02\,\text{mM}$). It follows that the evolution of the enzymatic rate versus total substrate concentration shows an acceleration in this region, which is particularly visible in Fig. 2. This upward curvature has been attributed by several authors to a positive effect of regulatory sites (for a review, see Ref. 27). On the contrary, when the

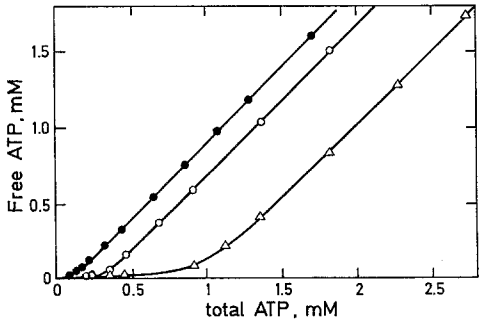

Fig. 4. Free ATP concentration, calculated according to Eq. (1), assuming $K_{as} = 0.02\,\text{mM}$, versus total ATP for different Mg^{2+} total concentrations. Same symbols as in Fig. 2.

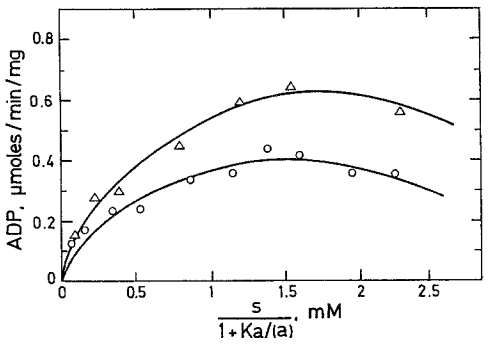

Fig. 5. Rate of ATP hydrolysis replotted versus $(s)/[1 + K_a/(a)]$. Same experiments as for Fig. 2.

reaction rate is plotted versus free ATP concentration, or more exactly versus $(s)/(1 + K_a/(a))$, the initial upward curvature disappears (Fig. 5). The curves obtained for any total magnesium concentration ought to be identical if the enzyme mechanism were completely described by Eq. (3). In fact, the curves are clearly different, which means that another phenomenon occurs. However, these curves are approximately homothetic, and the K_m's measured from them are of the same order: 0.1 and 0.4 mM. The determination of K_m is difficult. Indeed, the initial part of the curves is unworkable, because the $(s)/(1 + K_a/(a))$ ratio must be of the same order of magnitude or greater than K_m, which is only possible when (s_t) is greater than (a_t). The final part of the curves also cannot be used because of an inhibition of the reaction velocity, which can be attributed to excess of substrate, as will be shown below. When the total concentration of Mg^{2+} is too low (Fig. 2, curve $Mg_t^{2+} = 0.1\,\text{mM}$), these two conditions are not met and K_m cannot be determined.

Plots of the reaction rate versus total magnesium concentration at fixed total substrate can also be used to measure K_m. However, the low values of $(s)/(1 + K_a/(a))$ should be avoided (initial parts of the curves in Fig. 1), since v becomes proportional to $(s)/(1 + K_a/(a))$. The final parts of the curves of Fig. 1 are not considered either, because the rate is low and not accurate. For total substrate concentrations of 0.284, 0.914, and 2.71 mM, the K_m values are determined with sufficient accuracy in the middle part of the curves: 0.28, 0.13, and 0.21 mM, respectively. These values are in reasonable agreement with those obtained from Fig. 5 (0.1 and 0.4 mM).

If we accept 0.2 mM as a reasonable average for K_m, the maximum rate of ATP hydrolysis can be obtained from Eq. (5), $k = 0.95\,\mu\text{mol}\,\text{min}^{-1}\,\text{mg}^{-1}$.

The discrepancy between this value and the generally much higher values found by the phosphate release method, has been discussed previously.[14]

However, Eq. (3) does not account for the existence of a maximum in the reaction rate when the total substrate concentration is varied and the total magnesium concentration is held constant (as is seen in Fig. 2): the derivative $dv/d(s_t)$ does not reach zero for any finite value of (s_t). The occurrence of a maximum can be explained by the hypothesis of an inhibition of the reaction rate by the binding of a second molecule of substrate:[2,28]

$$EAS + S \rightleftarrows EAS_2,$$

$$\text{with dissociation constant } K_{m_2} = \frac{(eas)(s)}{(eas_2)}$$

The fixation of ATP on the enzyme could occur by two subsites: one binding the adenine moiety, the other, the phosphate groups. We propose that, when ATP is present in large excess, two molecules of ATP are bound per site on the enzyme, one on each of the two subsites, and that the hydrolysis mechanism is then inhibited. With this hypothesis, the reaction rate is (see Appendix 3)

$$v = \frac{k(e_t)}{1 + (K_m/s)(1 + K_a/(a)) + (s)/K_{m_2}} \qquad (6)$$

When the total substrate concentration is varied at fixed total magnesium concentration, the maximum rate is reached when $d(v)/d(s_t)$ or $d(1/v)/d(s_t) = 0$.

As seen in Fig. 2, the maximum occurs when $(s_t) > (a_t)$. Thus, $(s) \approx (s_t) - (a_t)$ and $(a) \approx K_{as}(a_t)/((s_t) - (a_t))$.

We have

$$v \approx \frac{k(e_t)}{1 + \dfrac{K_m}{(s_t) - (a_t)} + \dfrac{K_a K_m}{K_{as}(a_t)} + \dfrac{(s_t) - (a_t)}{K_{m_2}}}$$

$$\frac{d(1/v)}{d(s_t)} = \frac{-K_m}{((s_t) - (a_t))^2} + \frac{1}{K_{m_2}} = 0$$

$$((s_t) - (a_t))^2 = K_m K_{m_2} \qquad (7)$$

From the values of the maxima with $(a_t) = 0.33$ and 1 mM (Fig. 2), we have $K_{m_2} = 24$ mM. However, the determination of this constant is not precise because of the uncertainty in the positions of the maxima.

With these hypotheses, the maximum of the reaction rate, when the total concentration of magnesium is varied and the total concentration of substrate is held constant, is

$$\frac{d(1/v)}{d(a_t)} = \frac{K_m}{((s_t) - (a_t))^2} - \frac{K_a K_m}{K_{as}(a_t)^2} - \frac{1}{K_{m_2}} = 0$$

or

$$\frac{(a_t)^2}{((s_t) - (a_t))^2} = \frac{K_a}{K_{as}} + \frac{(a_t)^2}{K_m K_{m_2}} \qquad (8)$$

Equation (8) differs from Eq. (4) by the term $(a_t)^2/K_m K_{m_2}$, which is negligible compared to K_a/K_{as} when (a_t) does not exceed 0.2 mM (as for curves $(s_t) = 0.284$ mm and $(s_t) = 0.914$ mM, Fig. 1). In these cases, the calculation of K_a as above is valid. But, as the ratio $(a_t)^2/((s_t) - (a_t))^2$ is found to be quite constant for the four curves in Fig. 1, one must admit that the constant K_{m_2} is underestimated.

Inhibition by Sodium Ions

The experiments reported in Fig. 6 show that sodium ions inhibit ATPase activity and the percentage of inhibition, for the same concentration of total substrate, decreases with the concentration of total magnesium ions. The same phenomenon occurs with potassium ions (data not shown). These cations do not bind to ATP to any significant extent since the dissociation constant is 0.066–0.11 M for sodium and 0.069–0.10 M for potassium.[29] The affinity of ATP for Na^+ or K^+ is thus between three and four orders of magnitude smaller than for Mg^{2+}. Consequently the inhibition must be attributed to a binding of Na^+ or K^+ on the enzyme. If, for the sake of simplicity, we neglect the inhibition by excess of substrate, the system can be treated as follows:

$$E + I \rightleftarrows EI,$$

$$\text{with dissociation constant } K_i = \frac{(e)(i)}{(ei)}$$

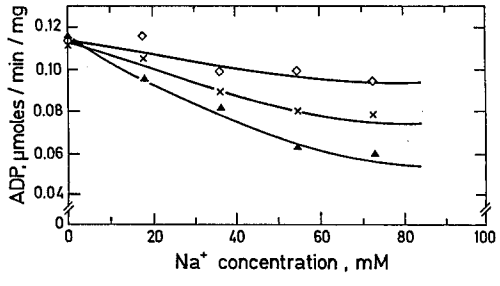

Fig. 6. Rate of ATP hydrolysis versus Na^+ concentration for different concentrations of Mg^{2+}. Final enzyme concentration: 10.2 µg/ml. Total ATP concentration 1.86 mM. (▲) 0.182 mM Mg_t^{2+}; (×) 0.454 mM Mg_t^{2+}; (◇) 0.91 mM Mg_t^{2+}.

where I is Na^+ or K^+

$$(e_t) = (e) + (ea) + (ei) + (x)$$

where (e) is the concentration of free enzyme, (ea) and (ei) are the concentrations of magnesium and sodium forms and (x) is the concentration of the EAS complex. We have $i \approx (i_t)$ = total inhibitor concentration, since high concentrations of sodium ions (20–80 mM) are required to observe inhibition. Then

$$(ei) = \frac{(e)(i_t)}{K_i}$$

and

$$(e_t) = (e)\left(1 + \frac{(i_t)}{K_i}\right) + (ea) + (x)$$

since

$$(e) = \frac{K_a(ea)}{(a)}$$

$$(e_t) = \frac{K_a(ea)}{(a)}\left(1 + \frac{(i_t)}{K_i}\right) + (ea) + (x)$$

From the above, we have (see Appendix 2)

$$v = \frac{k(e_t)}{1 + \left[1 + \frac{K_a}{(a)}\left(1 + \frac{(i_t)}{K_i}\right)\right]\frac{K_m}{(s)}} \qquad (9)$$

This model accounts for the experimental results shown in Fig. 7, where $1/v$ varies linearly with Na^+ concentration. Moreover, for the three concentrations of magnesium ion used and with the K_a, K_m, and k values determined above, the inhibition constant K_i is found to be 0.67, 0.56, and 0.62 mM respectively (mean value 0.62 mM).

DISCUSSION

In this work we have reinvestigated in a systematic manner the influence of ATP and ion concentrations on the rate of ATP hydrolysis by ϵ-depleted F_1 from spinach chloroplasts. The reason why we have used ϵ-depleted CF_1 and not native CF_1 is that the latter is naturally inhibited by its ϵ subunit and thus has a very low ATPase activity, which depends on the dissociation of the ϵ subunit from the complex and varies greatly, in a nonlinear manner, with CF_1 concentration. We have found, however, that at constant CF_1 concentration the shapes of the plots of ATPase activity versus Mg^{2+} concentration are identical to those obtained with ϵ-depleted CF_1 (data not shown).

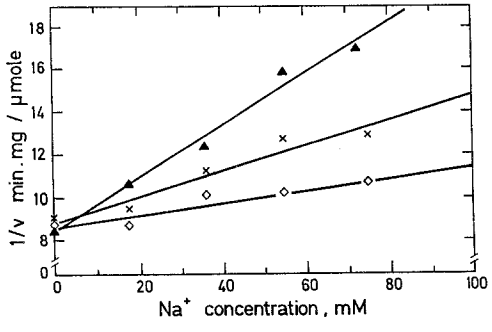

Fig. 7. Reciprocal of the rate of ATP hydrolysis versus Na^+ concentration. Same data as for Fig. 6.

We have shown that the experimental results can be explained by assuming a mechanism in which the enzyme must be activated by magnesium ion before hydrolyzing free ATP: the ascending parts of the curves in Fig. 1 are attributed to the activation of the enzyme by magnesium ion, and the descending parts to the depletion of the true substrate, i.e., free ATP, by formation of the MgATP complex. We have determined the values of different constants of the enzymatic reaction. It must be noted that they depend on the experimental conditions (pH, ionic strength) and on the value of the dissociation constant of MgATP (we have chosen the value of 0.02 mM, which corresponds to the most recent determinations under conditions similar to our enzymatic assays.[26]

We will now show that the classical mechanism, in which the free enzyme hydrolyzes the MgATP complex and is inhibited by magnesium ion, is unable to explain the totality of the experimental results. Indeed, if we write

$$E + A \rightleftarrows EA$$

where A is Mg^{2+} and K_a is the dissociation constant of the complex, assumed to be inactive, and

$$E + AS \underset{k'_{-1}}{\overset{k'_1}{\rightleftarrows}} EAS \xrightarrow{k'_2} \text{Products}$$

where AS is MgATP and

$$K'_m = \frac{k'_{-1} + k'_2}{k'_1}$$

we have (see Appendix 4)

$$v = \frac{k(e_t)}{1 + (1 + K_a/(a)) \cdot (K'_m K_{as}/K_a) \cdot 1/(s))} \qquad (10)$$

This relationship is similar to (3). The inhibition

constant K_a is equal to the activation constant of the first model (1.4 μM), and:

$$K'_m = \frac{K_m K_a}{K_{as}} = \frac{0.2\,\text{mM}}{0.02\,\text{mM}} \times 1.4\,\mu\text{M} = 14\,\mu\text{M}$$

The curves of reaction rate versus total substrate (Fig. 2) can be effectively explained by the following mechanism: the initial low value of the velocity is due to a very strong inhibition of the enzyme by magnesium, until an excess of substrate lowers the free magnesium concentration and reactivates the enzyme. This mechanism could also account for the general shape of the curves of reaction rate versus total magnesium concentration (Fig. 1): the increasing initial part is due to the formation of MgATP, and the decreasing part to inhibition of the enzyme by magnesium ion.

However, there is a contradiction between the K'_m value calculated for this mechanism from enzymatic data (14 μM) and the dissociation constant K_D^{MgATP} measured by the Hummel and Dreyer method and by a Scatchard plot under the same conditions, which was found to be 180 μM (Fig. 8). Indeed $K'_m = (k'_{-1} + k'_2)/k'_1$ ought to be superior to $K_D^{\text{MgATP}} = k'_{-1}/k'_1$. This discrepancy does not exist if we consider that ATP is the true substrate: K_m is 200 μM and K_D^{ATP}, measured from the same experiment (Fig. 8) by calculating free ATP, is 2.5 μM.

If we accept that Mg^{2+} inhibits the enzyme, it is logical to think that the Na^+ form of the enzyme, like the free form, is active. Assuming that these two forms have the same enzymatic constant toward MgATP, and that Na^+ competes with Mg^{2+} at the same sites

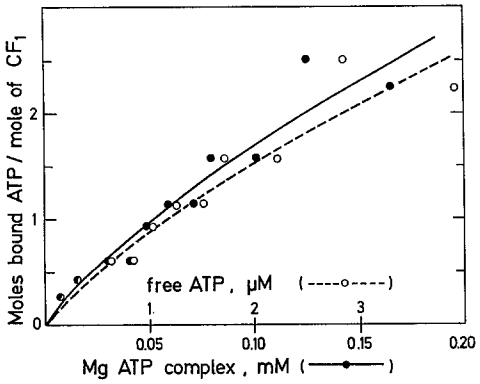

Fig. 8. Number of moles of ATP bound per mole of CF_1 (r) versus MgATP complex concentration (●) or free ATP concentration (○), measured by the Hummel and Dreyer method. Total magnesium ion concentration = 1 mM. Tightly bound ADP = 1.7 mol/mol CF_1. Other conditions described in the text.

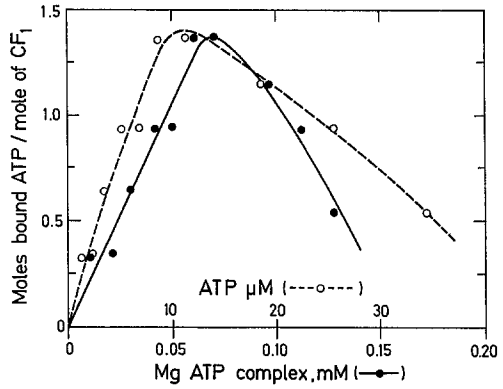

Fig. 9. Number of moles of ATP bound per mole of CF_1 (r) versus MgATP complex concentration (●) or free ATP concentration (○), measured by the Hummel and Dreyer method. Total magnesium ion concentration = 0.2 mM. Tightly bound ADP = 1.1 mol/mol CF_1. Other conditions described in the text.

on the enzyme, we obtain (see Appendix 5)

$$v = \frac{k(e_t)}{1 + \dfrac{K'_m}{(as)}\left(1 + \dfrac{(a)}{K_a}\dfrac{K_i}{K_i + (i_t)}\right)} \qquad (11)$$

where (i_t) is the total concentration of Na^+. According to this relationship, for the same (a) and (as) values, v would increase in the presence of Na^+. An activating effect of Na^+ would be predicted with these hypotheses, which is in contradiction with the experimental results. A more complex explanation could also be given, in which Na^+ would bind to the enzyme in a noncompetitive manner with Mg^{2+}.

Finally, the shape of the curve of r (moles of ATP bound per mole of enzyme) versus substrate concentration for 0.2 mM total magnesium concentration (Fig. 9) cannot be explained by the binding of the free enzyme with the MgATP complex: the r value tends toward zero when the MgATP concentration increases toward 0.2 mM, although it should be maximal. On the contrary, this phenomenon is well explained by our hypothesis: the binding decreases because of a lowering of the free magnesium ion concentration and thus of the Mg^{2+}–enzyme complex concentration.

We have neglected the contribution of the equilibrium $E + S \rightleftarrows ES$. There is no appreciable binding of ATP by CF_1, as measured by the Hummel and Dreyer method, in the absence of magnesium: $r = 0.2 \pm 0.2$ up to $(s) = 150\,\mu$M (data not shown). Nevertheless, the enzymatic rate is low but not zero (see Fig. 1). Traces of divalent cations in the water or in the buffer are perhaps responsible for this slight activation of the enzyme.

The equilibrium EA + AS $\rightleftarrows$ EA AS has also not been taken into account in this study. The Hummel and Dreyer method shows that the binding of ATP by CF_1 (r value) for the same concentration of total ATP below 0.1 mM is 1.5–2 times smaller with a total concentration of magnesium of 5 mM, compared to 1 mM (data not shown). This phenomenon is due to the depletion of free ATP when the total magnesium ion concentration is increased. It would be the same if MgATP were competing with free ATP on the enzyme–Mg^{2+} complex (EA). However, we cannot exclude the formation of a small amount of the EAAS complex. What is sure is that it does not dissociate into ADP since the rate of hydrolysis tends to nearly zero when the total magnesium concentration is increased.

Another hypothesis has been proposed which attributes the inhibitory effect of Mg^{2+} to the binding of ADP in the MgADP form at the catalytic sites.[5,31,32] This hypothesis was based essentially on the observation of a high enzymatic rate during a transient period (30 s to 2 min) after the start of the reaction, followed by the establishment of a constant value at a lower level. Our results are quite different since we observe neither a significant burst of rate nor a lag during the first minutes. The measured ADP values (the first one can be measured at 30 s) are, within the limit of our accuracy, aligned with the zero-time value (corresponding to ADP contained in ATP as impurity).[14] The slope of this line is taken as the initial rate and is constant up to a percentage of ATP hydrolysis of around 10%, during several minutes to more than one hour, depending on the experimental conditions.

Moreover, we think that the amount of ADP produced by ATP hydrolysis cannot influence strongly the hydrolysis rate by binding on the ATPase since its initial concentration is very low (the ADP content of ATP is of the order of 1%); it then increases linearly with time, several times during a period where the rate of ATP hydrolysis remains constant. It is known that ADP is inhibitory when added to the medium,[6,14,33] but the inhibition is only noticeable when its concentration is rather high, of the order of more than 20% of that of ATP. We thus think that ADP produced by hydrolysis cannot influence the initial rate.

Conversely, it seems that the transient rate observed by these authors[5,31,32] can be explained by our hypothesis: the totality of the catalytic sites of the activated CF_1 used by Guerrero *et al.*[5] are certainly filled (the final concentration of ATP in the activating medium is around 5 mM), and the initial rate is maximal. After dilution with the ATP regenerating medium, the concentration of ATP decreases, ATPase loses nucleotides, and the rate decreases. On the contrary, during incubation with magnesium, Mg ATP complex is formed, the free ATP concentration is low, only a few sites are filled, and the enzymatic rate is low. The $t^{1/2}$ of 1 min of the spectral changes of TF_1 mixed with nucleotides studied by Hisabori *et al.*[34] corresponding to the binding of nucleotides on the different sites, agrees with the duration of the transient rate. The reason why we do not observe such a phenomenon is presumably that our assays are performed at 37°C instead of 22°C in the work of Guerrero *et al.* Under our conditions, the equilibrium of fixation is more rapidly reached. These authors indeed noted that "an increase in temperature (50°C), ... reduces drastically the Mg^{2+}-induced inhibition of the steady-state rate."

CONCLUSION

In conclusion, we propose here a mechanism of action of the chloroplast ATPase involving first an activation of the enzyme by magnesium, followed by binding and hydrolysis of free ATP, whereas the classical theory considers that there is inhibition of the enzyme by magnesium ion and hydrolysis of the MgATP complex. To our knowledge, the explanation we propose has only been advocated in an early paper of Adolfsen and Moudrianakis concerning the coupling factor from *Alcaligenes faecalis*.[30]

Our data are based on HPLC separation and determination of ADP. No ATP regenerating system has been used in order to avoid any possible modification of free Mg^{2+} concentration by phosphoenolpyruvate. The enzymatic rates are defined with sufficient accuracy during several minutes and are proportional to the $CF_1 - \epsilon$ concentration, as shown in a previous publication.[14] The binding measurements are obtained chromatographically using the Hummel and Dreyer method, which allows us to determine low-affinity binding, since the complex is always in equilibrium with a constant concentration of ligand. It will be of great interest to reinvestigate the question of the actual substrates of similar nucleotide-binding enzymes, such as adenylate kinase or DNAses, in order to learn if they use free or metal-complexed substrates.

APPENDIX 1

Calculation of (a) and (s) from (a_t) and (s_t) (See Also Ref. 35)

$$(a_t) = (a) + (as)$$

$$(s_t) = (s) + (as)$$

$$K_{as} = \frac{(a)(s)}{(as)}$$

$$(a_t) = (a) + \frac{(a)(s)}{K_{as}} = (a)\left(1 + \frac{(s)}{K_{as}}\right)$$

$$(a_t) = \frac{K_{as}(as)}{(s)} \cdot \left(1 + \frac{(s)}{K_{as}}\right)$$

$$(a_t) = \frac{K_{as}}{(s)}[(s_t) - (s)]\left(1 + \frac{(s)}{K_{as}}\right)$$

$$= (s_t) + \frac{(s_t)K_{as}}{(s)} - K_{as} - (s)$$

$$(s)^2 + (s)[(a_t) - (s_t) + K_{as}] - K_{as}(s_t) = 0$$

$$s = \frac{-[(a_t) - (s_t) + K_{as}] \pm \sqrt{[(a_t) - (s_t) + K_{as}]^2 + 4K_{as}(s_t)}}{2}$$

In the same way,

$$(s_t) = (s) + \frac{(a)(s)}{K_{as}} = (s)\left(1 + \frac{(a)}{K_{as}}\right)$$

$$(s_t) = \frac{K_{as}(as)}{(a)} \cdot \left(1 + \frac{(a)}{K_{as}}\right)$$

$$(s_t) = \frac{K_{as}}{(a)}[(a_t) - (a)]\left(1 + \frac{(a)}{K_{as}}\right)$$

$$a^2 + a[(s_t) - (a_t) + K_{as}] - K_{as}(a_t) = 0$$

$$a = \frac{-[(s_t) - (a_t) + K_{as}] \pm \sqrt{[(s_t) - (a_t) + K_{as}]^2 + 4K_{as}(a_t)}}{2}$$

In both cases, there is only one positive (physically meaningful) solution.

APPENDIX 2

Calculation of the Enzymatic Rate with the Hypotheses That ATP Is the Substrate and Mg^{2+} is the Activator (See Also Ref. 36)

At the steady state, the concentration x of the complex EAS is constant:

$$\frac{d(x)}{dt} = -(k_2 + k_{-1})(x) + k_1(ea)(s) = 0$$

then

$$(x) = \frac{(ea)(s)k_1}{k_2 + k_{-1}}$$

and

$$(e_t) = (e) + (ea) + (x)$$

where $(e_t), (e)$, and (ea) are, respectively, the concentrations of the total enzyme, free enzyme, and magnesium bound enzyme.

The dissociation constant $K_a = (e)(a)/(ea)$. We then have

$$(e) = \frac{K_a(ea)}{(a)}$$

$$(e_t) = (ea)\left(1 + \frac{K_a}{(a)}\right) + (x)$$

$$(ea) = \frac{(e_t) - (x)}{1 + K_a/(a)}$$

$$(x) = \frac{k_1(s)((e_t) - (x))}{(k_2 + k_{-1})(1 + K_a/(a))}$$

$$(x)\left(1 + \frac{K_a}{(a)}\right)\left(\frac{k_2 + k_{-1}}{k_1}\right) + (s)(x) = (s)(e_t)$$

$$(x) = \frac{(e_t)}{1 + (1 + K_a/a)((k_2 + k_{-1})/k_1)(1/(s))}$$

if we write $(k_2 + k_{-1})/k_1 = K_m$ and k for maximum velocity, we obtain Eq. (3):

$$v = \frac{k(e_t)}{1 + (1 + K_a/(a))(K_m/(s))}$$

APPENDIX 3

Calculation of the Enzymatic Rate with the Hypothesis That the Binding of a Second Molecule of ATP Inhibits the Hydrolysis Mechanism

In addition to the above-mentioned equilibria, we have

$$\text{EAS} + \text{S} \rightleftarrows \text{EAS}_2, \qquad \text{with } K_{m_2} = \frac{(eas)(s)}{(eas_2)}$$

$$(e_t) = (e) + (ea) + (eas) + (eas_2)$$

$$= (ea)\left(1 + \frac{K_a}{(a)}\right) + (eas) + (eas_2)$$

$$= (eas)\left[1 + \frac{K_m}{(s)}\left(1 + \frac{K_a}{(a)}\right) + \frac{(s)}{K_{m_2}}\right]$$

$$v = \frac{k(e_t)}{1 + (K_m/(s))(1 + K_a/(a)) + ((s)/K_{m_2})}$$

APPENDIX 4

Calculation of the Enzymatic Rate with the Hypotheses That MgATP Is the Substrate and Mg^{2+} is the Inhibitor (See Also Ref. 36)

We consider the equilibria

$$E + A \rightleftarrows EA, \qquad \text{where A is } Mg^{2+}$$

with K_a = dissociation constant of the complex, assumed to be inactive.

$$E + AS \underset{k'_{-1}}{\overset{k'_1}{\rightleftarrows}} EAS \xrightarrow[k'_2]{} \text{Products},$$

where AS is MgATP

$$K'_m = \frac{k'_2 + k'_{-1}}{k'_1}$$

$$A + S \rightleftarrows AS,$$

where K_{as} = dissociation constant of AS

With the same definitions as in Appendix 2 and (x') for the concentration of the EAS complex, we have

$$(e_t) = (e) + (ea) + (x') = e\left(1 + \frac{(a)}{K_a}\right) + (x')$$

$$(e) = \frac{(e_t) - (x')}{1 + (a)/K_a}$$

The steady state is reached when $d(x')/dt = 0$:

$$-(k'_{-1} + k'_2)(x') + k'_1(e)(as) = 0$$

$$(x') = \frac{k'_1(e)(as)}{k'_{-1} + k'_2} = \frac{(e)(as)}{K'_m} = \frac{(e) \cdot (a) \cdot (s)}{K'_m K_{as}}$$

$$= \frac{(e_t) - (x')}{1 + (a)/K_a} \cdot \frac{(a) \cdot (s)}{K'_m K_{as}}$$

We have

$$(x') = \frac{(e_t)}{1 + K_{as}K'_m \cdot (1/(a) \cdot (s))(1 + (a)/K_a)}$$

and

$$v = \frac{k(e_t)}{1 + (K'_m/K_a)K_{as} \cdot (1/(s))(1 + K_a/(a))}$$

APPENDIX 5

Calculation of the Enzymatic Rate in the Presence of Na^+, with the Hypotheses That MgATP Is the Substrate and Mg^{2+} is the Inhibitor

We have the equilibria

$$E + A \rightleftarrows EA, \qquad \text{where A is } Mg^{2+}$$

K_a = dissociation constant of the complex EA assumed to be inactive,

$$E + I \rightleftarrows EI, \qquad \text{where I is } Na^+ \text{ (or } K^+)$$

K_i = dissociation constant of the complex EI assumed to be as active as E.

$$E + AS \rightleftarrows EAS \longrightarrow \text{Products},$$

concentration of EAS = (x)

$$EI + AS \rightleftarrows EIAS \longrightarrow \text{Products},$$

concentration of EIAS = (ix)

With the same definition as in Appendices 2 and 3, we have

$$(e) + (ea) + (ei) + (x) + (ix) = (e_t)$$

$$(e)\left(1 + \frac{(a)}{K_a} + \frac{(i)}{K_i}\right) + (x) + (ix) = (e_t)$$

If E and EI are as active toward AS, we have

$$(x) = \frac{(e)(as)}{K'_m}, \qquad (ix) = \frac{(ei)(as)}{K'_m}$$

$$(x) + (ix) = \frac{[(e) + (ei)](as)}{K'_m} = \frac{1}{K'_m}(as)(e)\left(1 + \frac{i}{K_i}\right)$$

$$(e) = \frac{(e_t) - (x) - (ix)}{1 + (a)/K_a + (i)/K_i}$$

$$\frac{(x) + (ix)}{1 + (i)/K_i} \cdot \frac{K'_m}{(as)}\left(1 + \frac{(a)}{K_a} + \frac{(i)}{K_i}\right) + (x) + (ix) = (e_t)$$

$$(x) + (ix) = \frac{(e_t)}{1 + \dfrac{K'_m}{(as)(1 + (i)/K_i)} \cdot \left(1 + \dfrac{(a)}{K_a} + \dfrac{(i)}{K_i}\right)}$$

$$v = \frac{k(e_t)}{1 + \dfrac{K'_m}{(as)}\left(1 + \dfrac{(a)}{K_a}\dfrac{K_i}{K_i + (i_t)}\right)}, \qquad \text{since } (i) \approx (i_t)$$

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REFERENCES

1. Ulrich, F. (1964). *J. Biol. Chem.* **239,** 3532–3536.
2. Adolfsen, R., and Moudrianakis, E. (1978). *J. Biol. Chem.* **253,** 4380–4388.
3. Gepshstein, A., Hochman, Y., and Carmeli, C. (1974). In *Proceedings of the Third International Congress on Photosynthesis,* September 2–6, Elsevier, The Netherlands, pp. 1189–1197.
4. Hochman, Y., Lanir, A., and Carmeli, C. (1976). *FEBS Lett.* **61,** 255–259.
5. Guerrero, K., Xue, Zhixiong, and Boyer, P. (1990). *J. Biol. Chem.* **265,** 16280–16287.
6. Malyan, A., and Makarov, A. (1977). *Biochemistry* **41,** 888–893 [translated from Biokhimiya **41,** 1087–1093 (1975)].
7. Anthon, G., and Jagendorf, A. (1983). *Biochim. Biophys. Acta* **723,** 358–365.
8. Zhou, J., and Boyer, P. (1992). *Biochemistry* **31,** 3166–3171.
9. Taussky, H., and Shorr, E. (1953). *J. Biol. Chem.* **202,** 675–685.
10. Avron, M. (1960). *Biochim. Biophys. Acta* **40,** 257–272.
11. Green, I., and Mommaerts, W. (1953). *J. Biol. Chem.* **202,** 541.
12. Carmeli, C., Lifshitz, Y., and Gutman, M. (1978). *FEBS Lett.* **89,** 211–214.
13. Pullman, M., Penefsky, H., Datta, A., and Racker, E. (1960). *J. Biol. Chem.* **235,** 3322–3329.
14. Berger, G., Girault, G., and Galmiche, J.M. (1990). *J. Liquid Chrom.* **13,** 4067–4080.
15. Younis, H., Winget, G., and Racker, E. (1977). *J. Biol. Chem.* **252,** 1814–1818.
16. Berger, G., Girault, G., Andre, F., and Galmiche, J. M. (1987). *J. Liquid Chrom.* **10,** 1507–1517.
17. Hummel, J., and Dreyer, W. (1962). *Biochim. Biophys. Acta* **63,** 530–532.
18. Scatchard, G. (1949). *Ann. N.Y. Acad. Sci.* **51,** 660–672.
19. Girault, G., Berger, G., Galmiche, J. M., and Andre, F. (1988). *J. Biol. Chem.* **263,** 14690–14695.
20. Berger, G., Girault, G., and Galmiche, J. M. (1989). *J. Liquid Chrom.* **12,** 535–551.
21. Burton, K. (1959). *Biochem. J.* **71,** 388-395.
22. O'Sullivan, W., and Perrin, D. (1964). *Biochemistry* **3,** 18–26.
23. Taqui Khan, M., and Martell, A. (1966). *J. Am. Chem. Soc.* **88,** 668–671.
24. Frey, C., and Stuehr, J. (1972). *J. Am. Chem. Soc.* **94,** 8898–8904.
25. Walaas, E. (1958). *Acta Chem. Scand.* **12,** 528–536.
26. Pecoraro, V., Hermes, J., and Cleland, W. (1984). *Biochemistry* **23,** 5262–5271.
27. Boyer, P. (1993). *Biochim. Biophys. Acta* **1140,** 215–250.
28. Girault, G., Galmiche, J. M., Michel-Villaz, M., and Thiery, J. (1973). *Eur. J. Biochem.* **38,** 473–478.
29. Phillips, R. (1966). *Chem. Rev.* **66,** 501–527.
30. Adolfsen, R., and Moudrianakis, E. (1973). *Biochemistry* **12,** 2926–2933.
31. Drobinskaya, I., Kozlov, I., Murataliev, M., and Vulfson, E. (1985). *FEBS Lett.* **182,** 419–424.
32. Milgrom, Y., and Boyer, P. (1990). *Biochim. Biophys. Acta* **1020,** 43–48.
33. Nelson, N., Nelson, H., and Racker, E. (1972). *J. Biol. Chem.* **247,** 6506–6510.
34. Hisabori, T., Yoshida, M., and Sakurai, H. (1986). *J. Biochem.* **100,** 663–670.
35. Malyan, A. (1981). *Photosynthetica* **15,** 474–483.
36. Dixon, M., and Webb, E. (1979). In *Enzymes,* 3rd edn., Longman Group Ltd., London, pp. 381–399.