Journal of Bioenergetics and Biomembranes, Vol. 30, No. 6, 1998

Cooperativity Between the Enzymatic Sites of F_1-ATPase Revisited by the Use of HPLC Methods

Gérard Berger,[1,2] Guy Girault,[1] and Jean-Luc Zimmermann[1]

Received April 29, 1998; accepted October 2, 1998

The fundamental question of the cooperativity between the enzymatic sites of F_1-ATPase is examined in the light of new measurements of the enzymatic rate of ATP hydrolysis by CF_1, the enzyme isolated from spinach chloroplasts. The experimental data, obtained with a chromatographic method, fit a model that involves two kinds of independent enzymatic sites working with metal-free ATP, with no need of cooperativity between the sites. Binding measurements between ADP or ATP and CF_1 by the chromatographic method of Hummel and Dreyer (1962) also support this conclusion. The present data and interpretation are in agreement with those reported recently (Reynafarje and Pedersen, 1996) which show that the first order rate constant of ATP hydrolysis by MF_1, the analogous enzyme from mitochondria, is virtually constant under experimental conditions involving either unisite or multisite hydrolysis of ATP. The present data and interpretation are discussed together with those reported previously, in particular with regard to the methods that were used to support the commonly accepted opposite viewpoint.

KEY WORDS: F_1-ATPase, enzymatic activity; nucleotide binding, cooperativity.

INTRODUCTION

F-type H^+-ATP synthases are membrane-bound enzymes present in chloroplasts, mitochondria, and certain bacteria that catalyze the phosphorylation of ADP to ATP using a transmembrane proton gradient as a source of energy. The F_1 part of the protein complex has the stoichiometry $\alpha_3\beta_3\gamma\delta\epsilon$, is hydrophilic, and can be isolated from the whole enzyme. The binding sites for nucleotides are located on this part, which retains, when isolated, the ability to drive the hydrolysis of ATP. The mechanism of this hydrolysis reaction has been studied for more than 30 years and, recently, the structures of the enzymes from bovine heart[1] and the $\alpha_3\beta_3$ subcomplex from the thermophilic bacterium *Bacillus* PS3[2] have been resolved by X-ray diffraction. The commonly accepted scheme for ATP hydrolysis involves a binding change mechanism[3,4] between the three nucleotide binding sites of the β subunits acting sequentially. The rotation of the γ subunit with respect to the rest of the enzyme has actually been observed under some particular conditions.[5]

An additional property, which has been reported for the F_1 enzyme, is the cooperativity between the enzymatic sites: positive cooperativity of catalysis and negative cooperativity of binding.[3,4,6,7] Numerous reports appear to be in favor of the theory of cooperativity between the nucleotide binding sites, however a number of reports,[8–11] including a recent one, demonstrate the Michaelian character of the F_1-ATPase.

F_1-ATPases can be isolated from a variety of different sources. The enzymes from chloroplasts, mitochondria, *Escherichia coli*, *Bacillus* PS3, and *Rhodospirillum rubrum* have been used in enzymatic studies. If qualitative differences exist between the enzymes from different sources, it may be thought that, given the similarity of their primary structures, all must share the main common properties such as

[1] CEA/Saclay, Département de Biologie Cellulaire et Moléculaire, Section de Bioénergétique, Bât. 532, F-91191 Gif-sur-Yvette, France.

[2] Author to whom correspondence should be addressed.

0145-479X/98/1200-0543$15.00/0

the subunit interactions, the nucleotide binding sites, and the mechanism of ATP hydrolysis, and that the discrepancy of the results cannot be reasonably ascribed to a difference in the material used. It must be noted, however, that the ATPase from chloroplasts, CF_1, is a latent enzyme that must be adequately activated. Different protocols have been reported by various groups, but the most frequently used treatment involves heating the enzyme in the presence of a large excess of ATP and dithiothreitol.[12]

Various experimental methods have been used to measure the enzymatic rate of F_1-ATPase. The first group of methods includes those in which the phosphate released from ATP was measured without separation from the other products,[13] after extraction of the complexed P_i,[14] precipitation of this complex,[15] or with radioactive ATP, by adsorption on charcoal of the unmodified ATP.[16] The release of protons due to hydrolysis of ATP has also been measured by means of a pH indicator [17] or by using a pH Stat.[18] The chemiluminescent method based on the luciferin/luciferase reaction is a sensitive method that can be used for monitoring ATP hydrolysis.[19] The majority of these studies have been carried out with an ATP-regenerating system, that involves phosphoenolpyruvate and pyruvate kinase, in order to maintain a constant substrate concentration. The reaction was monitored either by the release of phosphate [20] or by the decrease of the absorbance due to NADH in the presence of lactate dehydrogenase.[21]

The binding of nucleotides to F_1-ATPase has also been measured using different experimental methods. The isotope trapping method of Rose [22] has been used by Cross *et al.*[7] and Grubmeyer *et al.* [6] to determine the dissociation and association rates of nucleotides on ATPase and, therefore, the K_Ds for nucleotides on catalytic sites. The separation by rapid chromatography on Sephadex columns, as described by Penefsky,[23] has been widely applied for the determination of binding constants of nucleotides.[6,24–26] Several physical methods do not require separation of bound nucleotides. These include fluorescence emission of the CF_1–tetracycline complex [27] or of ethenoadenine ADP bound to CF_1,[28] which is modified by nucleotide binding, and the differential UV absorption spectroscopy of nucleotides, [26,29,30] which is based on the modification of the spectrum due to the hydrophobic conditions in the nucleotide binding pocket [30] or the circular dichroism of the ADP molecule, which is changed when bound to CF_1.[31] The fluorescence [32] or the UV-visible spectral changes [33] induced by binding of analogs of natural nucleotides like TNP ADP and TNP ATP have also been studied, as well as the modification of the fluorescence of the tryptophan probes incorporated near the nucleotide binding sites by site-directed mutagenesis[34–36] or the steady-state fluorescence intensity of maleimide attached to β-Cys-63 of CF_1.[37] The binding of nucleotides has also been measured using equilibrium dialysis, which is a method that is theoretically suitable in any situation, particularly for systems with low affinity.[24,30,33]

Besides the materials and the methods used for the enzymatic measurements, the conditions of the measurements may also be an important source of discrepancies. The ionic strength and the pH of the medium both act on K_m and V_m, as well as on the dissociation constants. In addition, numerous substances like bicarbonate and sulfite anions, DMSO, DNP, methanol, and octylglucoside have been shown to enhance the hydrolytic activity of CF_1 and have been routinely used in kinetic studies, although the precise effect on the kinetics of F_1 is unclear.

The effect of the divalent cations, especially Mg^{2+} and Ca^{2+}, must be examined separately. This is because these cations are normal constituents of the cell and also because they are able to modify the enzymatic rate by several orders of magnitude. The range of concentrations for these cations that is usually explored includes the micromolar and millimolar concentrations. In fact, because Mg^{2+} and Ca^{2+} form complexes with nucleotides, the important parameter for the enzymatic measurements appear to be the ratio of total ATP and Mg^{2+} concentrations $[ATP]_t/[Mg^{2+}]_t$. The dissociation constants of divalent metal cations with ATP are in the 10^{-4}–10^{-5} M range [38] and it is virtually unanimously accepted, but not quite demonstrated, that the Mg–ATP complex is the true substrate of F_1-ATPase.[39–41] The free Mg^{2+} cation is deduced to be an inhibitor, in order to explain the decrease of the activity when the Mg^{2+} concentration is progressively increased. We have challenged this interpretation, however, on the basis of the comparison of the binding and enzymatic data [42] and of the use of Mg^{2+} chelating agents with different dissociation constants.[43] Another interpretation has thus emerged for the mechanism of ATPase, with metal-free ATP being the true substrate of the enzyme and Mg^{2+} being an activator. This different view should certainly also have an influence on the interpretation of other experiments, especially those involving the cooperativity of nucleotide sites.

MATERIALS AND METHODS

Preparation of CF_1 and $CF_1 - \epsilon$

Chloroplast ATPase (CF_1) was prepared from spinach and extracted by a treatment with EDTA, sucrose, and chloroform [44] and then purified using DEAE-cellulose and Protein Pak DEAE 5 PW HPLC columns.[45] Experiments reported in the present study were performed with CF_1 depleted of its ϵ subunit ($CF_1 - \epsilon$): CF_1 in 20 m*M* Tris-SO_4, pH 8.5, was submitted to three cycles of freeze thawing and then centrifugated at 40,000 × *g* for 15 min. The resulting supernatant was injected on a Protein Pak DEAE 5 PW column (21.5 × 150 mm). Extensive washing of the column with 20 m*M* Tris-SO_4, pH 8.5 (1–2 h, 4 ml/min) led to partial fractionation of CF_1. By elution with an ammonium sulfate gradient (0–0.8 *M* in 40 min), the β subunit, $CF_1 - \delta$, and $CF_1 - \epsilon$ complexes could be separated.[45]

Measurement of Activity

The enzymatic activity was determined using an HPLC method based on the separation of ADP and ATP and the quantitation of ADP formed. This method has proved to be rapid, simple, and without artifacts.[46] ATPase activity was measured at 37°C in 75 m*M* Tris-SO_4 buffer, pH 8.5, containing variable concentrations of ATP and Mg^{2+} (routinely 1 and 0.2 m*M*, respectively.) Aliquots of the reaction mixture were directly injected on a TSK DEAE 2 SW column (4 × 300 mm). This operation takes about 5 s (closure of the injection port and of two valves). However, mixture of the aliquot with the elution buffer can be estimated to be effective in less than 1 s. It can be assumed that the enzymatic reaction is then immediately stopped by the low pH of the buffer (KH_2PO_4 0.1 *M*, NaCl 0.25 *M*, pH 4.3). The enzyme precipitates in this buffer and bound nucleotides are released. ADP was separated from ATP in about 3 min, at the elution rate of 1.2 ml/min, with a resolution better than 2. The concentration of the released ADP was measured by the height of the absorption peak at 260 nm, compared to a calibration curve established under the same conditions. The concentrations of ATP and ADP standards were calculated assuming $E^{M}_{1\,cm} = 15{,}400$ and were corrected for the traces of AMP and ATP present in ADP and of ADP in ATP, measured by HPLC. The amount of ADP released by ATPase-driven ATP hydrolysis increased linearly with the time of action of the enzyme in the reaction mixture, up to a percentage of hydrolysis of about 10%. The initial enzymatic rate is well defined for a period of at least several minutes. After this time, the rate decreases because of the consumption of ATP and of the inhibition due to released ADP. No lag time or burst of activity was observed under these experimental conditions, that is, when $CF_1 - \epsilon$ was previously dialyzed against 75 m*M* Tris-SO_4, pH 8.5, even in the first minute (aliquots were taken every 10 s and frozen in liquid N_2 before analysis). The initial concentration of ADP in the reaction mixture was determined by extrapolation to zero time and corresponded exactly to that brought as an impurity by ATP. Endogenous ADP or ATP that are tightly bound to CF_1 or $CF_1 - \epsilon$ were measured after acid denaturation of the protein using the same chromatographic method. These amounts were negligible in the conditions of the measurements of the enzymatic activity. It was verified that the quantity of ADP measured by HPLC was completely independent on the quantity of ATP from which it was separated (in the range 0–2.5 × 10^{-6} *M* ADP, in the absence or presence of 10^{-3} *M* ATP). The enzymatic rate varies linearly with the quantity of $CF_1 - \epsilon$, contrary to CF_1, which dissociates into $CF_1 - \epsilon$ and inhibitory ϵ at low concentration.

Measurement of Nucleotide Binding

For the binding studies, we have used the chromatographic method of Hummel and Dreyer.[47] The advantage of this method is that the complex does not dissociate during the course of the chromatography, even if the affinity constant is low, since the complex is always in equilibrium with the free ligand. The initial method of Hummel and Dreyer has been extended to HPLC by Sebille *et al.* [48] for the study of the binding of different drugs on albumin and, in our previous reports, we have applied it to the determination of the number of binding sites of CF_1 and of some of its subunits or partial complexes.[42,43,49] A chromatographic column separating the protein from the ligand (here a TSK DEAE 2 SW anion-exchange column, or a TSK G 2000 SW gel filtration column) is equilibrated with a buffer containing a fixed concentration of ligand (nucleotide). A known quantity of protein is injected, which binds a fraction of ligand, depending on the ligand concentration and the dissociation constant. The bound ligand migrates with the protein and the local trough of the nucleotide concentration migrates, as a

negative peak, with the ligand rate. Hummel and Dreyer have proposed an internal calibration by successive additions of known amounts of ligand to the same quantity of protein. The size of the ligand peak varies linearly with the excess of ligand over the quantity contained in the same volume of eluent. The bound quantity corresponds to the excess that anneals the peak. This bound quantity depends only on the ligand concentration on the column at the time of the injection and on the dissociation constants of the complexes. It does not depend on the volume of the mixture and is rigorously proportional to the injected amount of protein. By this method, only reversible sites can be measured, those that can be filled within a few seconds. Slower bindings give rise to trailings. In the case of CF_1, the 1 to 1.5 tightly bound ADP molecules, which are found on the isolated molecule, do not interfere with the binding measurements. This is because these tightly bound nucleotides do not dissociate when the buffer does not contain any nucleotide. These 1 to 1.5 nucleotide molecules add to the extrapolated number of reversible sites determined by HPLC to yield the total number of binding sites. With ADP, in the presence of 1 m*M* Mg^{2+}, this total number is always 6 $\pm$ 0.5 sites, as for ATP under the same conditions of Mg^{2+} concentrations.[49] For ATP, however, the total number of six binding sites is not always reached when the conditions of binding are not favorable and the extrapolation is not reliable. With CF_1 containing the ϵ subunit, no appreciable hydrolysis of ATP occurs during the time (<20 s) of the preparation and the injection of the mixture.[50] In this method, the size of the negative peak corresponding to ligand binding is not modified by events occurring after the binding of ATP on the enzyme and the withdrawal of the corresponding quantity of ligand from the eluent. Thus, only ATP binding is really measured and not ADP that would correspond to hydrolysis of ATP. Using this method, quasilinear Scatchard plots are obtained, which are characteristic of an apparently unique class of sites.

The binding and enzymatic studies are measured using 75 m*M* Tris buffer, pH 8.5, and with variable concentrations of Mg^{2+} and ATP. We have avoided the use of any activating substances such as detergents, solvents, reducing agents or others, that could modify the binding and hydrolysis mechanisms.

RESULTS

Enzymatic Data

The ATPase rate of $CF_1-\epsilon$ has been followed in the range of total ATP concentrations from 0.7×10^{-6} *M*–10^{-3} *M*, with a total Mg^{2+} concentration of 1 m*M*. With the present method of measurement, the enzymatic rate cannot be determined with accuracy for concentrations of ATP lower than 0.7×10^{-6} *M*. This lower limit does not actually correspond to what is usually called unisite conditions ($[ATP]_t/[CF1]_t < 1$). However, only about one nucleotide site of ATPase is filled with ADP or ATP: the enzyme used here has been shown to contain 0.94 mole ADP per mole of CF_1, and no ATP. The binding of ATP to CF_1 is proportional to the concentration of ATP for low concentrations (0.3 site/mole for 10^{-5} *M* $[ATP]_t$, and 1 m*M* $[Mg^{2+}]_t$, 75 m*M* Tris-SO_4) (Fig. 1). Consequently, at the lowest level of ATP used in this work, only about one nucleotide site is filled with nucleotides and far less than one site is filled with ATP.

In the low range of concentrations ($[ATP]_t$ from 0.7 to 6 μM), the evolution of the enzymatic rate can be compared with that of a Michaelian enzyme (Fig. 2). For concentrations ranging from 6 to 50 μM, the rate varies quite linearly with $[ATP]_t$ (Fig. 3). Beyond 50 μM and up to 600 μM, a new Michaelian type curve is observed (Fig. 4), which is the commonly described evolution, the deviations at lower concentrations being negligible at this level of enzymatic rates.

In order to account for all the data, especially those obtained at low substrate concentrations, we have used a model involving two types of catalytic sites that hydrolyze ATP independently from each other and with two different K_ms and V_ms. A similar model has been proposed to explain the enzymatic data obtained for TF_1.[51]

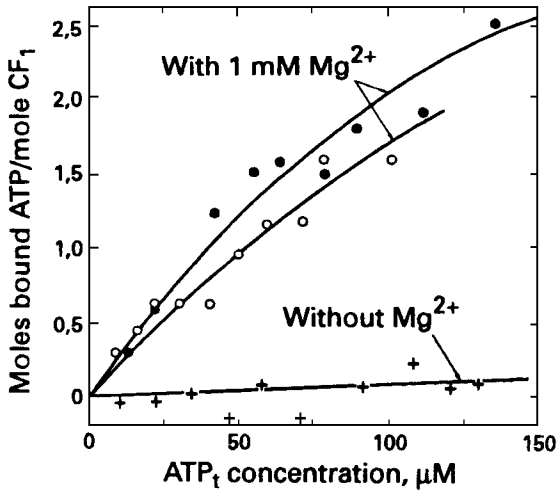

Fig. 1. Number of moles of bound ATP per mole of CF_1 versus total ATP concentration, in the presence of 1 m*M* Mg^{2+} or without Mg^{2+}.

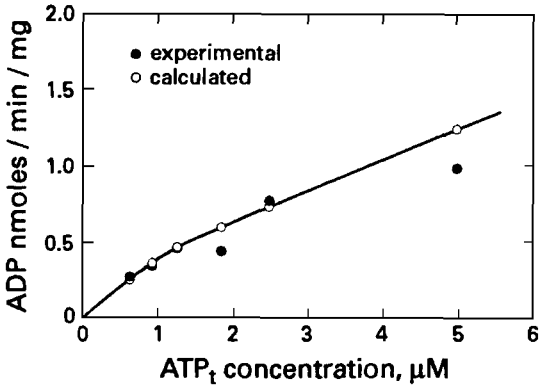

Fig. 2. Enzymatic rates of $CF_1 - \epsilon$. Conditions described in Table I Closed symbols, experimental data; open symbols, calculated as $v_1 + v_2$, $v_1 = 0.36/(1 + 0.018/s)$ nmol ADP/mg/min and $v_2 = 114/(1 + 12/s)$ nmol ADP/mg/min; s is the free-metal ATP in μmol/l.

The enzymatic rate of a single type of sites has been shown to be described by the equation:

$$v = V_m/1 + \left(1 + \frac{K_a}{a}\right)\frac{K_m}{s} + \frac{s}{K'_m} \qquad (1)$$

where s is the metal free substrate concentration, a is the free Mg^{2+} concentration, K_a is the activation constant (1.4×10^{-6} M; see Ref. 42), K_m is the Michaelis constant, and K'_m is the dissociation constant of a complex containing two molecules of substrate per site (24 mM; see Ref. 42).

Under the present experimental conditions, the $[Mg^{2+}]_f$ concentration is larger than the $[ATP]_f$ concentration, so that Eq. (1) reduces to:

$$v \cong V_m/(1 + K_m/s)$$

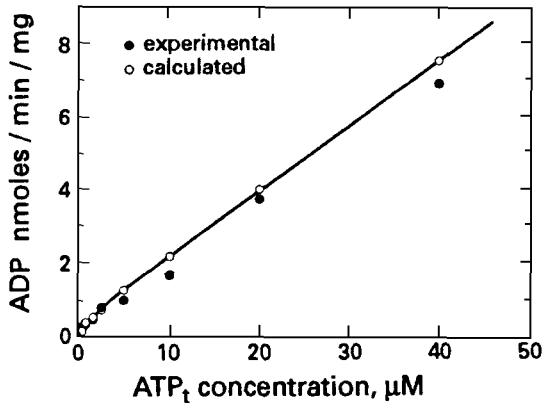

Fig. 3. See legend, Fig. 2.

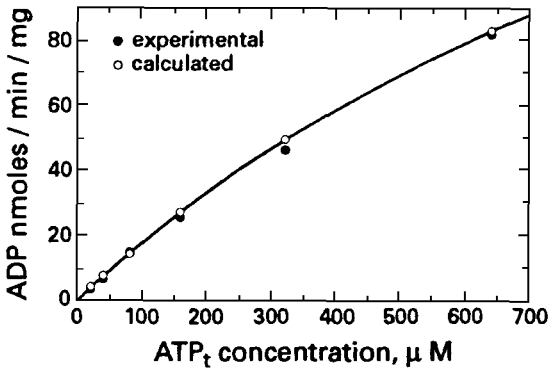

Fig. 4. See legend, Fig. 2.

When two types of sites are active, this becomes:

$$v \cong [V_{m1}/(1 + K_{m1}/s)] + [V_{m2}/(1 + K_{m2}/s)] \qquad (2)$$

K_{m2} and V_{m2} corresponding to high substrate concentration, can be determined by neglecting the first term of the relationship. We found $V_{m2} = 114$ nmoles/min/mg and $K_{m2} = 12$ μM (Fig. 5).

At low substrate concentration, V_{m1} and K_{m1} can be calculated by subtracting from v the contribution from $[V_{m2}/(1 + K_{m2}/s)]$ (Table I). We found $V_{m1} = 0.36$ nmoles/min/mg and $K_{m1} = 18$ nM (Fig. 6).

The trace representing the evolution of Eq. (2) with the parameters determined above fits well the experimental points (Figs. 2–4). Thus the present model with two independent categories of sites can be considered as adequately describing the experimental data, without the need of cooperativity between the sites.

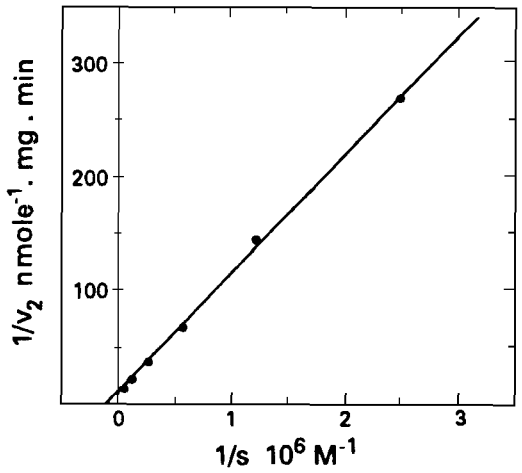

Fig. 5. Lineweaver-Burk representation of the enzymatic rate for high $[ATP]_f$ concentrations. The extrapolation yields $V_{m2} = 114$ nmol ADP/mg/min and $K_{m2} = 12$ μM.

Table I. The Calculated Metal-Free ATP and Free Mg^{2+} Concentrations, Measured Enzymatic Rate, and Calculated Rates for Two Kinds of Sites for $[Mg^{2+}]_t$ = 1 m*M* and variable $[ATP]_t$ Concentrations[a]

$[ATP]_t$ (μ*M*)	$\left[\frac{\text{Free-metal ATP}}{}\right]$ (μ*M*)	[Free Mg^{2+}] (μ*M*)	v Experimental enzymatic rate (nmol ADP/min/mg)	v_1 Calculated enzymatic rate first type of site (nmol ADP/min/mg)	v_2 Calculated enzymatic rate second type of site (nmol ADP/min/mg)	$v_1 + v_2$ (nmol ADP/min/mg)
0.625	0.0125	999.4	0.278	0.147	0.119	0.266
0.94	0.01891	999.1	0.336	0.184	0.179	0.363
1.25	0.02503	998.8	0.461	0.209	0.237	0.446
1.87	0.03747	998.1	0.439	0.243	0.355	0.598
2.5	0.0501	997.5	0.772	0.264	0.474	0.738
5	0.1005	995	0.984	0.305	0.947	1.25
10	0.198	990.2	1.68	0.33	1.85	2.18
20	0.3998	980.4	3.71	0.344	3.68	4.02
40	0.8156	960.8	6.94	0.352	7.25	7.60
80	1.699	921.7	14.9	0.356	14.14	14.50
160	3.704	843.5	25.8	0.358	26.9	27.3
320	9.026	689	46.5	0.359	48.9	49.3
640	31.13	391.1	81.6	0.359	82.3	82.6

[a] Measurements were performed at 37°C, using CF_1 – ε containing 0.94 mol/mol of residual ADP at a concentration of 8.4 μg/ml in Tris buffer, 75 m*M* pH 8.5. $K_{DMg\text{-}ATP}$ = 0.019 m*M*.

When the total ATP concentration is further increased beyond $[Mg^{2+}]_t$, the concentration of metal-free ATP steeply increases and if the enzyme is not yet completely saturated at the equivalence point, then an acceleration of the enzymatic rate is observed (Fig. 7). As it will be discussed below, this kind of stimulation has been attributed to cooperativity between the enzymatic sites.[52,53]

Finally, at even higher metal-free ATP concentrations, the hydrolysis rate passes through a maximum and then decreases. We have ascribed this phenomenon to substrate inhibition, due to the binding of a second molecule of substrate in the enzymatic site.[42]

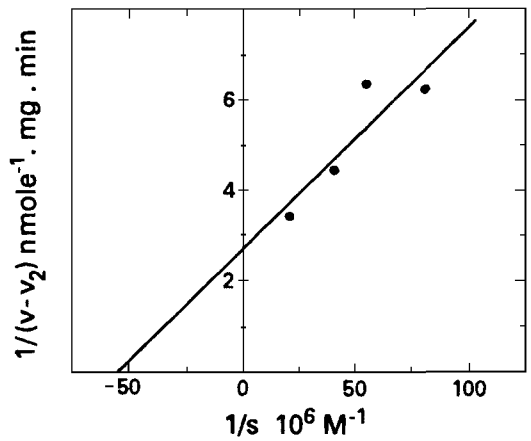

Fig. 6. Lineweaver-Burk representation of the enzymatic rate minus v_2, for low $[ATP]_t$ concentrations. The extrapolation yields V_{m1} = 0.36 nmol ADP/mg/min and K_{m1} = 18 n*M*.

Nucleotide Binding Data

Figures 8 and 9 show the Scatchard plots, corresponding to the binding of nucleotides, measured by the chromatographic method of Hummel and Dreyer. The curves obtained for ADP and ATP are both linear, reflecting that no cooperativity between the sites

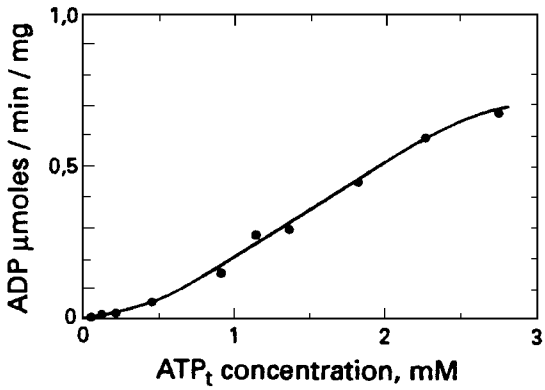

Fig. 7. Enzymatic rate of CF_1 – ε (17.9 μg/ml), when the total ATP concentration exceeds the total $[Mg^{2+}]_t$ concentration (1 m*M*).

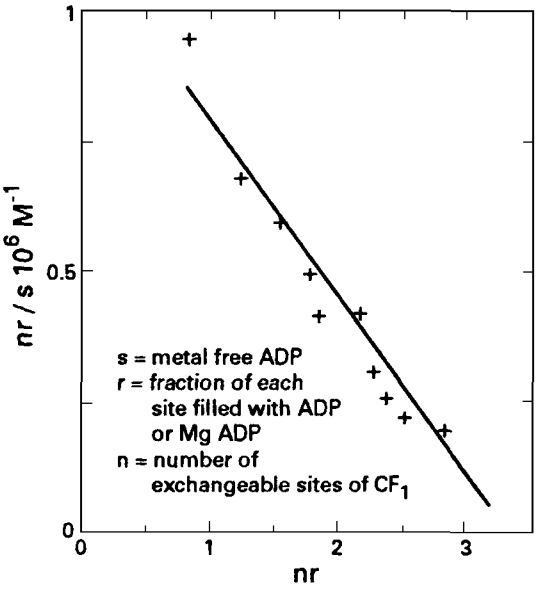

Fig. 8. Scatchard plot of CF_1–ADP binding measurements. The measurements were performed in 75 m*M* Tris buffer, pH 8.5, and 1 m*M* $[Mg^{2+}]_f$.

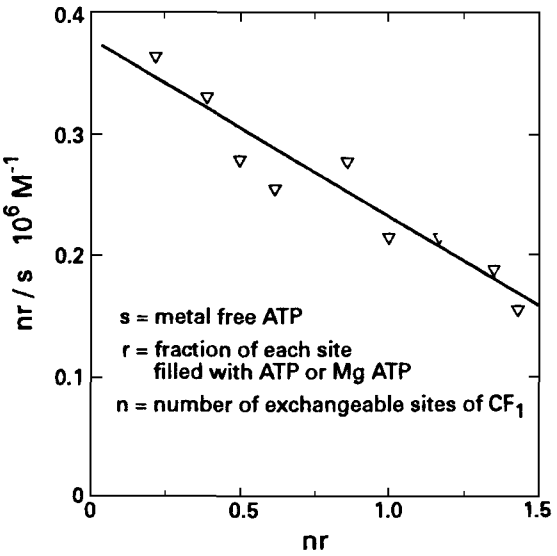

Fig. 9. Scatchard plot of CF_1–ATP binding measurements. The measurements were performed in 75 m*M* Tris buffer, pH 8.5, and 0.4 m*M* $[Mg^{2+}]_f$.

occurs under the conditions used in the present experiment.

The fraction r of each site supposed to be filled with both metal-free and metal-complexed nucleotide is given by the relationship:[43]

$$\frac{1}{r} = 1 + (1 + K_a/a) \times K_{D,EMg\text{-}ANP}/s \times [1/1 + (a \times K_{D,EMg\text{-}ANP})/ \times (K'_{D,EMg\text{-}Mg\text{-}ANP} \times K_{D,Mg\text{-}ANP})]$$

where s is the concentration of metal-free ANP, a is the concentration of free Mg^{2+}, K_a is the dissociation constant of the Mg–enzyme complex, K_a = ([E][Mg]/[EMg]), $K_{D,Mg\text{-}ANP}$ is the dissociation constant of the Mg–ANP complex, $K_{D,Mg\text{-}ANP}$ = ([Mg][ANP]/[Mg-ANP]), $K_{D,EMg\text{-}ANP}$ is the dissociation constant of the free-metal ANP–Mg activated enzyme complex, $K_{D,Mg\text{-}ANP}$ = ([EMg][ANP]/[EMg-ANP]), and $K'_{D,EMg\text{-}Mg\text{-}ANP}$ is the dissociation constant of the Mg–ANP–Mg activated enzyme complex, $K'_{D,EMg\text{-}Mg\text{-}ANP}$ = ([EMg][Mg-ANP]/[EMg-Mg-ANP]).

Under the conditions of the measurements, a_t is in large excess over the total nucleotide concentration ($a \approx a_t$), and the term (K_a/a) is negligible. The Scatchard plot is a straight line and the slope is $-[1/K_{D,EMg\text{-}ANP} + a/(K'_{D,EMg\text{-}Mg\text{-}ANP} \times K_{D,Mg\text{-}ANP})]$

Consequently, a plot of these slopes as a function of mean a (or a_t) allows the determination of $K_{D,EMg\text{-}ANP}$ and $K'_{D,EMg\text{-}Mg\text{-}ANP}$ (Table II).[43]

DISCUSSION

Only a few authors have described the mechanism of F_1-ATPase as belonging to the Michaelian type: Pedersen [9,54] and Ebel and Lardy [8] for the enzyme in the presence of bicarbonate. The thermophilic enzyme TF_1 has also been described as Michaelian for concentrations of ATP up to 20 μ*M* [10]. More recently, Reynarfaje and Pedersen [11] have shown that only one type of nucleotide site was working under experimental conditions involving unisite as well as multisite hydrolysis, and with the same rate constant. In their work, the enzyme is shown to exhibit typical Michaelis-Menten kinetics characterized by a single V_{max} and a single K_m of 57 μ*M* (expressed as Mg-ATP). No cooperativity between the sites is observable.

Table II. Dissociation Constants of the CF_1–Mg Complex with ADP, ATP, and Their Mg^{2+} Complexes

$K_{D,Mg\text{-}ADP}$: 5–15 μ*M*	$K'_{D,EMg\text{-}Mg\text{-}ADP}$: 64 μ*M*
$K_{D,EMg\text{-}ATP}$: 14 μ*M*	$K'_{D,EMg\text{-}Mg\text{-}ATP}$: 180 μ*M*

The interpretation that is proposed here for the present results is in accord with their conclusion with regard to the noncooperativity between the sites: indeed, the model used above that best fits our data does not need this hypothesis. In this model, however, two kinds of independent nucleotide sites are considered, with quite different V_ms and K_ms, with only one being observable (V_m = 114 nmoles/min/mg) in the usual range of total ATP concentrations, i.e., from 10^{-3} to 10^{-5} M, the other one having a V_m 300 times smaller. This difference with the results of Reynafarje and Pedersen[11] can probably be ascribed to the difference in the nature of the ATPases. In fact, for TF_1, the enzyme from the thermophilic bacterium *Bacillus* PS3, the ratio between the V_m of the two kinds of sites is 3 (Ref. 51) and it is understandable that it could also be different in the case of the mitochondrial F_1-ATPase, in such a way that the sites with lower rate could not be observable.

At high substrate concentration, the specific activity of the $CF_1 - \epsilon$ used in the present study is quite low compared with results published in the literature. It can be noted however, (Table III) that the measured activity largely depends on the nature of the ATPase. Activities reported for $CF_1 - \epsilon$ are about two orders of magnitude lower than those for MF_1 and for TF_1, intermediate activities are found. On the other hand, as mentioned above, the activity of $CF_1 - \epsilon$ can be enhanced by DTT,[55] octylglucoside,[56] sulfite,[56] ethanol,[52] DMSO, etc. These effectors, which are definitely not physiological substances, have purposely not been used in the present study to avoid possible perturbation on the enzyme mechanism. In addition, because of the different physiological roles of MF_1 and CF_1, there is no reason why the maximal activities of these two ATPases should be comparable.

The binding of nucleotides to CF_1, measured by the chromatographic method of Hummel and Dreyer, does not show obvious signs of cooperativity. In fact, the Scatchard plots are generally linear.[43, 50] This does not mean that only one kind of binding site is present. In CF_1, between 1 and 1.5 nucleotide sites are irreversibly filled with ADP, which does not dissociate from CF_1, even when the chromatographic eluent does not contain nucleotides. Thus these nucleotide sites do not interfere with the binding measurements. The binding of nucleotides to low rate enzymatic sites is possibly too slow to be completely measured by this method. Under certain conditions, we have observed on the chromatogram a trailing near the negative peak of optical density that corresponds to the binding of the nucleotide. In addition, when the enzyme is incubated for 5 min with ADP prior to the injection on the column, an additional 0.5 site is observed to be filled with nucleotide and the Scatchard plot can be treated with a two-sites type model.[49] However, this experiment cannot be performed with ATP, which would be partially hydrolyzed.

The enzymatic data shown above are interpreted using a classical model with two kinds of nucleotide binding sites and no cooperativity between the sites. However, many authors have interpreted their enzymatic data using the double hypothesis of positive cooperativity of catalysis and negative cooperativity of binding. Some points relevant to these hypotheses are discussed below.

The experimental results that are usually taken as evidence for the cooperativity of catalysis between the nucleotide sites are those of Grubmeyer *et al.* [6] and Cross *et al.*[7] The turnover of catalysis that is measured under unisite conditions (10^{-4} s^{-1}) is compared with that measured under multisite conditions (300–600 s^{-1}) and the ratio between the two ($\approx 10^6$) is considered as reflecting the catalytic cooperativity. It is of note, however, that if there is a relative agreement on the turnover value under multisite conditions,

Table III. Specific Activity of $CF_1 - \epsilon$ in Present Study, Compared with Literature

Nature of the ATPase	Specific multisite activity (μmoles ADP/min/mg)	Turnover number (multisite activity) s^{-1}	Turnover number (unisite activity) s^{-1}	Method of measurement	References
MF_1	100	600	10^{-2}–10^{-1}	Luciferin–luciferase	11
	100	600	3.6×10^{-4}	^{32}P release ATP regenerating	6, 7
TF_1	12	80		Chromatography	51
$CF_1 - \epsilon$	3.8	25		^{32}P release	55 (Table 1)
	4	27		^{32}P release	52 (Fig. 6)
	1.6	11		^{32}P release	56 (Table 1)
	0.1–1	0.7–7	2.4×10^{-3}	Chromatography	This work and 42

this is not the case for that in unisite conditions. Grubmeyer *et al.* [6] have measured the latter by the dissociation of ADP from the F_1–ADP–P_i complex as a function of time. The ADP is separated by centrifugation on a Sephadex column and expressed as a percentage of the initial radioactivity. The results reported by these authors [6,7] may, however, be biased by a number of factors. First, the isotope trapping method of Rose,[22] which is used in the experiment, has its own limitations, and it is necessary, as indicated by the author, that suitable controls for a mixing artefact be included. Second, the frequently used ATP regenerating method,[20,21] involving phosphoenolpyruvate at high concentration, which certainly modifies by complexation the concentration of free Mg^{2+}, [57] is probably not suitable for studies of the effect of substrate or Mg^{2+} concentrations. It is of note that Mal'yan and Makarov [58] have observed large differences in the measured enzymatic rate whether the regenerating system was used or not. The discrepancies were ascribed to the removal of the inhibitory ADP formed, but this interpretation may be erroneous given the low percentage of hydrolyzed ATP. Third, it is hard to explain why the initial rate of release of ADP (measured below 5 min) under unisite conditions is several times larger than the rate that is measured 10 minutes after, the latter being taken as the true rate (see ref. 6, Fig. 5) In addition, the different curves reflecting the enzymatic activity obtained by the dilution of the same ATP/F_1 mixture are not superimposed. In fact the determination of the unisite turnover rate was revised later (4×10^{-3} s^{-1}; ref. 25), but this value is several orders of magnitude lower than those reported by Gresser *et al.* [3] or by Reynafarje and Pedersen [11] (9.6 and 0.1 s^{-1}, respectively). In addition, the latter authors argue that the measurement of the rate of hydrolysis reported by Grubmeyer *et al.* [6] is not direct, but based on the dissociation of ADP bound to F_1 after catalysis has taken place and that this could simply reflect the displacement of inhibitory ADP bound to a catalytic site when ATP concentration is increased.

A quite different approach has been used by Andralojc and Harris, [52, 53] who have also concluded that there is positive cooperativity of catalysis between the sites. These authors, using CF_1 in the presence of Ca^{2+}, have observed a stimulation of the activity as a function of the total ATP concentration within the range 15–50 μM and they attributed this phenomenon to the cooperativity between the nucleotide sites. However, at a 1 m*M* metal ion concentration, the concentration of metal-free ATP, increases sharply when the total ATP concentration exceeds 1 m*M*. If, as we propose, ATP is the true substrate and if the enzyme is not fully saturated at the equivalent point, then the enzymatic rate should display a sharp increase as a function of total ATP or Mg-ATP concentration (but not, versus free-metal ATP). In fact, with 1 m*M* Mg^{2+}, the acceleration begins for lower concentrations of total ATP ($\approx$ 500 μM; see Fig. 7 and Ref. 42) and for Ca^{2+}, which has a larger dissociation constant with ATP, the acceleration occurs at even lower concentrations of total ATP. Then, the stimulation observed by Andralojc and Harris [52,53] can be explained by this phenomenon, without referring to the hypothesis of cooperativity.

The ^{18}O exchange measurements between ATP and H_2O performed by Hutton and Boyer [59,60] have led their authors to eliminate the possibility of the participation of two pathways of hydrolysis (unisite and multisite) and this result was considered as readily explained by catalytic cooperativity between the sites. However, if the substrate of ATPase is actually metal-free ATP, then the relative participation of two types of independent sites can vary, as a function of total ATP concentration, as follows:

$$v \cong [V_{m1}/(1 + K_{m1}/s)] + [V_{m2}/(1 + K_{m2}/s)]$$

At very low total ATP concentrations we have:

$$s = K_{D,\text{Mg-ATP}}.[\text{Mg-ATP}]/[\text{Mg}]$$

$$\cong K_{D,\text{Mg-ATP}}.[\text{ATP}]_t/[\text{Mg}]_t$$

When s is low, but sufficiently larger than K_{m1},

$$v_1 = V_{m1}$$

$$v_2 \cong \frac{V_{m2}s}{K_{m2}} \cong \frac{V_{m2}}{K_{m2}} K_{D,\text{Mg-ATP}}.\frac{[\text{ATP}]_t}{[\text{Mg}]_t}$$

One can imagine particular conditions for which the first mechanism would be predominant, for a given range of $[ATP]_t$ concentrations, for instance, if:

$$V_{m1} \gg \frac{V_{m2}}{K_{m2}} K_{D,\text{Mg-ATP}}.\frac{[\text{ATP}]_t}{[\text{Mg}]_t}$$

while the second mechanism would be predominant at higher concentration of $[ATP]_t$. Two types of independent sites could then exist, each being predominant in a particular range of total ATP concentrations and yet the ^{18}O exchange measurement would reveal a unique mechanism at low $[ATP]_t$ concentrations. The authors have performed their measurements at 3 and 6 μM, which may be out of the transition range where the two mechanisms are both acting.

The possibility of fitting both binding and rate coefficients to the whole data of initial velocity, bound ADP and ^{18}O exchange between H_2O and released P_i, has been given as evidence for the model of the alternating three-sites mechanism, i. e., catalytic cooperativity between three sites, because such a fit cannot be obtained with an alternating two-sites model.[3] The system may, however, be artificially parametrized, due to the large number, 18, of independent constants in the model that were modified using manual iterations to obtain the best fit to the data.

CONCLUSION

The chromatographic methods used here for measuring the enzymatic rate of CF_1 ATPase or the binding of nucleotides are simple methods based on the separation of ligands or reaction products from the protein. They appear to be less susceptible to artifacts than indirect methods often used to address these questions. $CF_1 - \epsilon$ was used in the present study as active ATPase, which is better defined than the DTT-treated and-heated enzyme. No exogenous activating agents such as DTT, octylglucoside, methanol, DMSO, or sulfite were used in the enzymatic measurements, which may perturb the mechanism and are certainly not physiological substances. Under the present conditions, the rates are relatively low (<1 μmole/min/mg). The present data can be interpreted by the classical Michaelis Menten mechanism, without the need of the additional hypothesis of cooperativity of catalysis. This conclusion is in agreement with that reached recently by Reynafarje and Pedersen,[11] who have demonstrated that the first rate constant of hydrolysis of ATP by mitochondrial ATPase remains constant under both unisite and multisite conditions. The binding data obtained by the chromatographic method of Hummel and Dreyer[47] (linear Scatchard plots; Ref. 43) also indicate no cooperativity of binding. This simple model has the advantage to be more easily verified and to better predict phenomena than do complicated mechanisms that involve the influence of regulatory sites or cooperativity between catalytic sites. This also shows that the mechanism by which ATP is synthesized and hydrolyzed in biological systems needs much further investigation.[54]

ACKNOWLEDGMENTS

We wish to thank Ms. Jeanine Le Cruër and Véronique Mary for their skillful assistance in preparing CF_1.

REFERENCES

1. Abrahams, J., Leslie, A., Lutter, R., and Walker, J. (1994). *Nature* **370**, 621–628.
2. Shirakira, Y., Leslie, A., Abrahams, J., Walker, J., Ueda, T., Sekimote, Y., Kambara, M., Saika, K., Kagawa, Y., and Yoshida, M. (1997). *Structure* **5**, 825–836.
3. Gresser, M., Myers, J., and Boyer, P. (1982). *J. Biol. Chem.* **257**, 12030–12038.
4. Boyer, P. (1993). *Biochim. Biophys. Acta* **1140**, 215–250.
5. Noji, H., Yasuda, R., Yoshida, M., and Kinosita, K. (1997). *Nature* **386**, 299–302.
6. Grubmeyer, C., Cross, R., and Penefsky, H. (1982). *J. Biol. Chem.* **257**, 12092–12100.
7. Cross, R., Grubmeyer, C., and Penefsky, H. (1982). *J. Biol. Chem.* **257**, 12101–12105.
8. Ebel, R., and Lardy, H. (1975). *J. Biol. Chem.* **250**, 191–196.
9. Pedersen, P. (1976). *J. Biol. Chem.* **251**, 934–940.
10. Yohda, M., and Yoshida, M. (1987). *J. Biochem.* **102**, 875–883.
11. Reynafarje, B., and Pedersen, P. (1996). *J. Biol. Chem.* **271**, 32546–32550.
12. Lien, S., and Racker, E. (1971). *Methods Enzymol.* **23**, 547–555.
13. Taussky, H., and Shorr, E. (1953). *J. Biol. Chem.* **202**, 675–685.
14. Avron, M. (1960). *Biochim. Biophys. Acta* **40**, 257–272.
15. Sujino, Y., and Miyoshi, Y. (1964). *J. Biol. Chem.* **239**, 2360–2364.
16. Girbes, T., Cabrer, B., and Modolell, J. (1979). *Methods Enzymol.* **59**, 353–362.
17. Carmeli, C., Lifshitz, Y., and Gutman, M. (1978). *FEBS Lett.* **89**, 211–214.
18. Green, I., and Mommaerts, W. (1953). *J. Biol. Chem.* **202**, 541–549.
19. Lundin, A., and Baltscheffsky, M. (1978). *Methods Enzymol.* **57**, 50–56.
20. Pullman, M., Penefsky, H., Datta, A., and Racker, E. (1960). *J. Biol. Chem.* **235**, 3322–3329.
21. Stiggall, D., Galante, Y., and Hatefi, Y. (1979). *Methods Enzymol.* **55**, 308–315.
22. Rose, I. (1980). *Methods Enzymol.* **64**, 47–59.
23. Penefsky, H. (1977). *J. Biol. Chem.* **252**, 2891–2899.
24. Kironde, F., and Cross, R. (1987). *J. Biol. Chem.* **262**, 3488–3495.
25. Cunningham, D., and Cross, R. (1988). *J. Biol. Chem.* **263**, 18850–18856.
26. Hisabori, T., and Sakurai, H. (1984). *Plant Cell Physiol.* **25**, 483–493.
27. Girault, G., and Galmiche, J.-M. (1978). *FEBS Lett.* **95**, 135–139.
28. Girault, G., and Galmiche, J.-M. (1977). *Eur. J. Biochem.* **77**, 501–510.
29. Hisabori, T., and Mochizuki, K. (1993). *J. Biochem.* **114**, 808–812.
30. Hisabori, T., Yoshida, M., and Sakurai, H. (1986). *J. Biochem.* **100**, 663–670.
31. Girault, G., Galmiche, J.-M., Michel-Villaz, M., and Thiery, J. (1973). *Eur. J. Biochem.* **38**, 473–478.
32. Geczi Digel, J., and McCarty, R. (1995). *Biochemistry* **34**, 14482–14489.
33. Hisabori, T., Muneyuki, E., Okada, M., Yokohama, K., Mochizuki, M., and Yoshida, M. (1992). *J. Biol. Chem.* **267**, 4551–4556.
34. Weber, J., Wilke Mounts, S., Lee, R., Grell, E., and Senior, A. (1993). *J. Biol. Chem.* **268**, 20126–20133.
35. Weber, J., Wilke Mounts, S., Grell, E., and Senior, A. (1994). *J. Biol. Chem.* **269**, 11261–11268.

36. Weber, J., Bowman, C., Wilke Mounts S., and Senior, A. (1995). *J. Biol. Chem.* **270**, 21045–21049.
37. Mills, D., Seibold, S., Squier, T., and Richter, R. (1995). *Biochemistry* **34**, 6100–6108.
38. Phillips, R. (1966). *Chem. Rev.* **66**, 501–527.
39. Ulrich, F. (1964). *J. Biol. Chem.* **239**, 3532–3536.
40. Gepshtein, A., Hochman, Y., and Carmeli, C. (1974). In *Proceedings of the Third International Congress on Photosynthesis (Avron, M., Ed.)*, Elsevier, Amsterdam, The Netherlands, pp. 1189–1197.
41. Hochman, Y., Lanir, A., and Carmeli, C. (1976). *FEBS Lett.* **61**, 255–259.
42. Berger, G., Girault, G., Galmiche, J.-M., and Pezennec, S. (1994). *J. Bioenerg. Biomemb.* **26**, 335–346.
43. Berger, G., Girault, G., Pezennec, S., and Zimmermann, J.-L. (1998). *J. Liquid Chromatogr. Rel. Technol.* **21**, 1925–1955.
44. Younis, H., Winget, G., and Racker, E. (1977). *J. Biol. Chem.* **252**, 1814–1818.
45. Berger, G., Girault, G., André, F., and Galmiche, J.-M. (1987). *J. Liquid Chromatogr* **10**, 1507–1517.
46. Berger, G., Girault, G., and Galmiche, J.-M. (1990). *J. Liquid Chromatogr.* **13**, 4067–4080.
47. Hummel, J., and Dreyer, W. (1962). *Biophys. Biophys. Acta* **63**, 530–532.
48. Sebille, B., Thuaud, N., and Tillement, J. P. (1978). *J. Chromatogr.* **167**, 159–170.
49. Girault, G., Berger, G., Galmiche, J.-M., and André, F. (1988). *J. Biol. Chem.* **263**, 14690–14695.
50. Berger, G., Girault, G., and Galmiche, J.-M. (1989). *J. Liquid Chromatogr.* **12**, 535–551.
51. Pezennec, S., Berger, G., Andrianambinintsoa, S., Radziszewski, N., Girault, G., Galmiche, J.-M., and Bauerlein, E. (1995). *Biochim. Biophys. Acta* **1231**, 98–110.
52. Andralojc, P., and Harris, D. (1990). *Biochim. Biophys. Acta* **1016**, 55–62.
53. Andralojc, P., and Harris, D. (1994). *Biochim. Biophys. Acta* **1184**, 54–64.
54. Pedersen, P. (1996). *J. Bioenerg. Biomemb.* **28**, 389–395.
55. Galmiche, J. M., Girault, G., Berger, G., Jacquot, J. P., Miginiac-Maslow, M., and Wollman, M. (1990). *Biochimie* **72**, 25–32.
56. Sokolov, M., and Gromet-Elhanan, Z. (1996). *Biochemistry* **35**, 1242–1248.
57. Norby, J. (1970). *Acta Chem. Scand.* **24**, 3276–3286.
58. Mal'yan, A., and Makarov, A. (1976). *Biochemistry* **41**, 888–893.
59. Hutton, R., and Boyer, P. (1979). *J. Biol. Chem.* **254**, 9990–9993.
60. Boyer, P. (1989). *FASEB J.* **3**, 2164–2178.