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Unisite ATP hydrolysis by soluble *Rhodospirillum rubrum* F_1–ATPase is accelerated by Ca^{2+}

Ernesto Maldonado [a], Georges Dreyfus [a,*], José J. García [a,1], Armando Gómez-Puyou [b], Marietta Tuena de Gómez-Puyou [a]

[a] *Departamento de Genética Molecular, Universidad Nacional Autónoma de México, Apartado Postal 70-243, Mexico D.F. 04510, Mexico*

[b] *Departamento de Bioquímica, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Apartado Postal 70-243, Mexico D.F. 04510, Mexico*

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Abstract

At saturating concentrations of ATP, soluble F_1 from the *Rhodospirillum rubrum* (RF_1) exhibits a higher rate of hydrolysis with Ca^{2+} than with Mg^{2+}. The mechanisms involved in the expression of a higher catalytic activity with Ca^{2+} were explored by measuring the ATPase activity of RF_1 at substiochiometric concentrations of ATP (unisite conditions). At a ratio of 0.25 [γ-^{32}P]ATP per RF_1, the enzyme exhibited a 50 times higher hydrolytic rate with Ca^{2+} than with Mg^{2+}. The rate of [γ-^{32}P]ATP binding to RF_1 was in the same range with the two divalent metal ions. Centrifugation–filtration of RF_1 exposed to substoichiometric [γ-^{32}P]ATP concentrations and Mg^{2+} through Sephadex columns yielded an enzyme that contained [γ-^{32}P]ATP and [^{32}P]phosphate in a stoichiometry that was close to one. In the presence of Ca^{2+}, the eluted enzyme did not contain [γ-^{32}P]ATP nor [^{32}P]phosphate. This indicated that the rate of product release was faster with Ca^{2+} than with Mg^{2+}. It was also observed that the ratio of multisite to unisite hydrolysis rates was of similar magnitude with both divalent cations. This suggests that they do not affect differently the cooperative mechanisms that may exist between catalytic sites. In consequence, the higher ATPase activity of RF_1 in presence of Ca^{2+} strongly suggests that the retention time of products is decreased in the presence of this cation. © 1998 Elsevier Science B.V.

Keywords: ATPase, F_1-; Unisite ATP hydrolysis; Ca^{2+}; Mg^{2+}; Product release; (*Rhodospirillum rubrum*)

1. Introduction

The ATP synthase of energy transducing membranes catalyzes the synthesis of ATP from ADP and P_i (for reviews, see [1,2]). The energy for the synthesis of ATP is provided by electrochemical H^+ gradients derived from electron transport that is initiated by substrate oxidation, or by light in photosynthetic organisms. The ATP synthase of the photosynthetic bacterium *Rodospirillum rubrum* has been extensively studied [3–6]. The enzyme from *R. rubrum* is similar to ATP synthases from other sources; it consists of a membrane moiety (F_0) whose function is to channel H^+ to a portion of the ATP synthase known

* Corresponding author. Fax: +52 5 616 2282; E-mail: gdreyfus@ifcsun1.ifisiol.unam.mx

[1] Present address: Instituto Nacional de Cardiología ''Ignacio Chávez'', Departamento de Bioquímica, Juan Badiano 1, Mexico D.F. 14080, Mexico.

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as F_1, the site where the catalytic events that lead to ATP synthesis take place. The latter segment has been isolated from chromatophores of *R. rubrum* (RF_1) and its properties and structure have been studied in several laboratories [6–9].

One of the most apparent characteristics of soluble F_1 is its ability to catalyze hydrolysis of ATP through a process that is Mg^{2+} dependent. The ATPase activity of RF_1 is also supported by Mg^{2+}, but at variance with for example mammalian mitochondrial F_1, its ATPase activity with Ca^{2+} is strikingly higher than with Mg^{2+} [7]. This is not unique to RF_1. F_1 from *Chromatium* [10], chloroplast [11], turnip mitochondria [12], *Rhodobacter sphaeroides* [13], *Bacillus subtillis* [14], *Micrococcus luteus* [15], and *Streptomyces lividans* [16], and other organisms also exhibit a higher ATPase activity with Ca^{2+} than with Mg^{2+}. Thus, this characteristic of F_1 seems to be rather widespread in ATP synthases of energy transducing membranes. This is rather peculiar, particularly if it is considered that light-driven ATP synthesis in chromatophores from *R. rubrum* or chloroplast membranes, is supported by Mg^{2+}, but not by Ca^{2+} [17,18]. This has raised a number of questions on how the hydrolytic events that occur in soluble RF_1 are related to ATP synthesis in intact chromatophores.

In this work, the mechanisms that lead to the expression of a higher ATPase activity of RF_1 in presence of Ca^{2+} were explored. The purpose of the experiments was to gain further information on some of the factors that operate in the kinetics of the enzyme. Specifically, we studied ATP hydrolysis under conditions in which ATP concentration is lower than that of RF_1 (unisite hydrolysis). As shown by various authors [19–22], under unisite conditions, only the catalytic site with the highest affinity for ATP carries out hydrolysis. The following is the accepted sequence of the reactions of unisite ATP hydrolysis

$$F_1 + ATP \Leftrightarrow F_1 \cdot ATP \Leftrightarrow F_1 \cdot ADP \cdot P_i$$
$$\Leftrightarrow F_1 \cdot ADP + P_i \Leftrightarrow F_1 + ADP$$

The results show that in presence of Ca^{2+}, unisite ATP hydrolysis is many-fold higher than with Mg^{2+} and that this is consequence of a higher rate of product release from the high affinity catalytic site.

2. Materials and methods

All non-radioactive reagents were purchased from Sigma. [^{32}P]P_i was from New England Nuclear; this was used for the preparation of [γ-^{32}P]ATP according to reference [23]; its specific activity was $2–3 \times 10^6$ cpm/nmol. Soluble RF_1 was prepared from *R. rubrum* strain S1. Bacteria were grown photosynthetically in the medium described by Sistrom [24]. Chromatophores were isolated from *R. rubrum* harvested in the logarithmic phase of growth [25]. Soluble RF_1 was extracted from chromatophores by the chloroform method and purified according to Norling et al. [8]. RF_1 dissolved in 50 mM Tris–HCl, 1 mM $MgCl_2$ and 15% glycerol, pH 7.5 at a concentration of about 20 mg/ml was stable for months at -70°C. Before use, the enzyme was thawed and passed through a column of Sephadex G-50 by centrifugation [26] equilibrated with the reaction medium without ATP. The molecular weight of RF_1 (384 kDa) was deduced from its nucleotide sequence [4].

2.1. Multisite hydrolysis

Multisite Ca^{2+}- and Mg^{2+}-ATPase activities of RF_1 were assayed in 25 mM Tris–acetate pH 8.0, 30 mM K-acetate, 3 mM $CaCl_2$ or $MgCl_2$ and 3 mM ATP. P_i formed was measured colorimetrically [27].

2.2. Unisite [γ-^{32}P]ATP hydrolysis and cold-chase experiments

In the standard method for measuring unisite [γ-^{32}P]ATP hydrolysis, 50 μl of RF_1 at a concentration of 2 μM in a mixture of 40 mM MES, 0.25 mM KH_2PO_4 and 2 mM $MgCl_2$ or 2 mM $CaCl_2$ pH 8.0 was mixed with 50 μl of 0.5 μM of [γ-^{32}P]ATP. At the desired times, the reaction was arrested with 300 μl of cold trichloroacetic acid (TCA) 6% final concentration. Carrier Ca^{2+}- or Mg^{2+}-ATP (4 mM, final concentration) were added to the arrested mixtures. The final volume was completed to 0.5 ml. [^{32}P]P_i was extracted twice with butyl acetate after formation of the phosphomolybdate complex as described elsewhere [28]. The amount of [γ-^{32}P]ATP

that remained in the water phase was used to calculate the amount of [γ-^{32}P]ATP hydrolyzed. In cold-chase experiments [19], 100 μl of non-radioactive Ca^{2+}- or Mg^{2+}-ATP (4 mM final concentration) were added to RF_1 undergoing unisite [γ-^{32}P]ATP hydrolysis. When Ca^{2+} was used, the reaction was allowed to proceed for 6 s; with Mg^{2+} the reaction was arrested after 1 min. The amount of [γ-^{32}P]ATP that was not hydrolyzed was determined as described above.

2.3. Determination of [γ-^{32}P]ATP bound to RF_1 by the hexokinase-glucose trap method

[γ-^{32}P]ATP binding to RF_1 under unisite conditions was determined by the hexokinase-glucose trap method [29]. For these experiments, RF_1 was incubated with Ca^{2+} or Mg^{2+} in the standard mixture for unisite [γ-^{32}P]ATP hydrolysis, except that the final volume was 40 μl. At the desired times, the samples were supplemented with 300 μl of a mixture at pH 8.0 that contained 20 units of hexokinase, 10 mM glucose and 10 mM $MgCl_2$; the reactions were arrested 30 s later with HCl (1.3 N, final concentration). The activity of hexokinase added was around 5×10^3-folds higher than the hydrolytic activity of RF_1 at saturating ATP concentrations. To determine the amount of [γ-^{32}P]ATP that had been converted into glucose 6-[^{32}P]phosphate, the samples were placed in boiling water (92°C) for 30 min. Subsequently, [^{32}P]P_i was extracted twice as described above, and the radioactivity in the aqueous phase was determined; this was considered to correspond to the amount of glucose 6-[^{32}P]phosphate formed. From the data, the rate of [γ-^{32}P]ATP binding was calculated according to reference [30]. Prior to the experiments two assays were carried out: (i) In the experiments with Ca^{2+}, Mg^{2+} had to be added with hexokinase in order to support hexokinase action; experiments in the absence of RF_1 showed that under these conditions, hexokinase converted around 97% of [γ-^{32}P]ATP into glucose 6-[^{32}P]phosphate in less than 2 s; (ii) the effect of heat treatment on [γ-^{32}P]ATP and glucose 6-[^{32}P]phosphate was determined; it was observed that heat treatment produced cleavage of more than 97% of [γ-^{32}P]ATP; and that on the average 98% of glucose 6-[^{32}P]phosphate was heat resistant.

2.4. Determination of [γ-^{32}P]ATP and [^{32}P] phosphate bound to RF_1 by the centrifugation–filtration method

In other experiments, the unisite mixture was centrifuged through Sephadex G-50 columns. The eluate was received in 100 μl of 12% TCA, an aliquot was used to determine total radioactivity. Another identi-

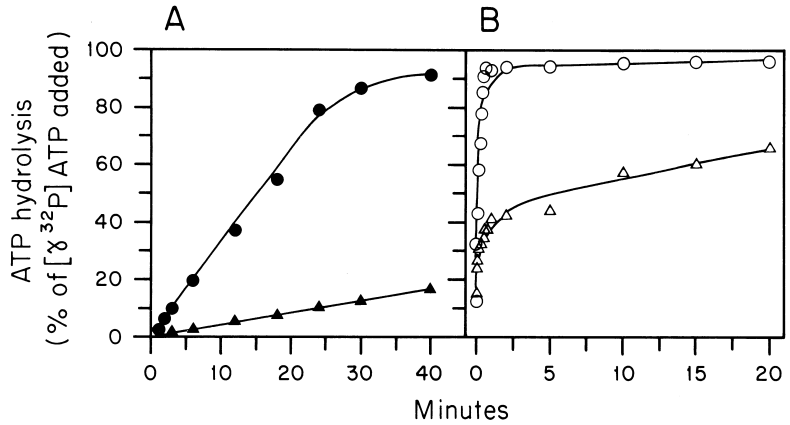

Fig. 1. Time course ATP hydrolysis by RF_1 at saturating (A) and substiochiometric (B) ATP concentrations. In (A), the reaction was carried out with 3 mM ATP and 3 mM of Mg^{2+} (▲) or Ca^{2+} (●); the reaction was initiated by adding 12 μg (▲) or 2 μg (●) RF_1 to the reaction medium (final volume = 100 μl) as indicated under Section 2. At the times indicated, the reactions were arrested and the amount of P_i formed was determined. Unisite ATPase activity (B) was started by mixing of equal volumes of RF_1 (2 μM) and [γ-^{32}P]ATP (0.5 μM) in the unisite standard buffer, in the presence of 2 mM of Mg^{2+} (△) or Ca^{2+} (○); at the times shown the reactions were arrested with TCA, and the amount of [γ-^{32}P]ATP that remained was determined.

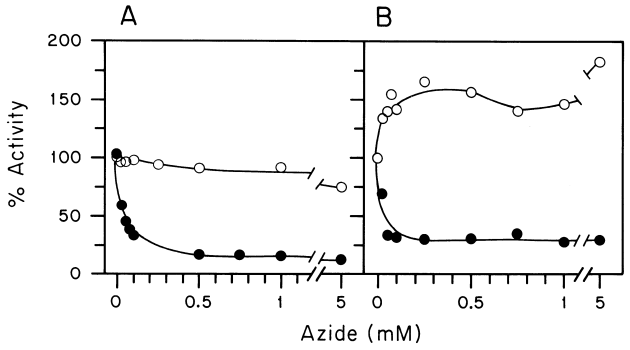

Fig. 2. Effect of different concentrations of azide on ATP hydrolysis by RF_1 under multisite (●) and unisite (○) conditions in presence of Ca^{2+} (A) or Mg^{2+} (B). The experimental mixtures were as in Fig. 1 except that azide was added at the indicated concentrations. Under unisite conditions, (○) represent the fraction of [γ-^{32}P]ATP hydrolyzed after 8 s of incubation with Ca^{2+} (panel A) or after 5 min of incubation with Mg^{2+} (panel B). In the absence of azide, the fractions of hydrolyzed [γ-^{32}P]ATP, were 0.18 and 0.13 mol [γ-^{32}P]ATP/mol RF_1 with Ca^{2+} and Mg^{2+}, respectively. Under multisite conditions (●), without azide, the ATPase activity (100%) was 6 and 0.1 μmol/min/mg with Ca^{2+}, and Mg^{2+}, respectively.

cal sample was extracted twice with butyl acetate after formation of the phosphomolybdate complex. The radioactivity in the aqueous phase was considered as [γ-^{32}P]ATP bound to RF_1. The difference between total radioactivity and [γ-^{32}P]ATP was equal to [^{32}P]P_i bound to RF_1. Protein was determined, according to Smith et al. [31], in eluates of identical samples, except that these were received in 100 μl of 10% SDS.

3. Results

The ATPase activity of RF_1 was measured at saturating and substoichiometric concentrations of ATP (Fig. 1(A) and (B), respectively) in presence of Ca^{2+} and Mg^{2+}. In confirmation of previous data [7] ATP hydrolysis was higher with Ca^{2+} under multisite conditions. Under unisite conditions in presence of Mg^{2+}, there was a rapid initial burst of [γ-^{32}P]ATP hydrolysis that was followed by a relatively slow phase of hydrolysis that continued until [γ-^{32}P]ATP was exhausted (Fig. 1(B)). This pattern has been observed with F_1 from various sources [19,20,32]. With Mg^{2+} the rapid phase reflects binding and splitting of [γ-^{32}P]ATP at the high affinity catalytic site, where an equilibrium between [γ-^{32}P]ATP and ADP and [^{32}P]P_i is established. The slow phase of hydrolysis is consequence of a slow rate of product dissociation from the catalytic site [33].

The time course of unisite ATP hydrolysis by RF_1 in the presence of Ca^{2+} differed markedly from that observed with Mg^{2+} (Fig. 1(B)). With Ca^{2+}, there was a continuous relatively rapid rate of [γ-^{32}P]ATP

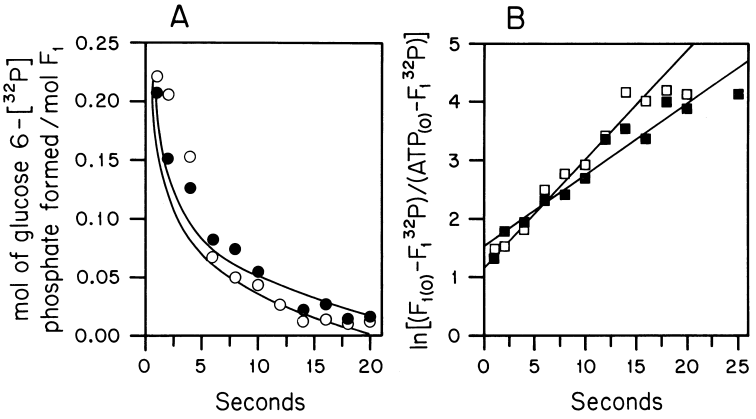

Fig. 3. Accesibility of [γ-^{32}P]ATP to hexokinase in RF_1 incubated under unisite conditions. In (A), [γ-^{32}P]ATP was incubated under unisite conditions with Mg^{2+} (●) or Ca^{2+} (○). At the times shown, hexokinase (20 units) and 10 mM glucose was added. In the tubes incubated with Ca^{2+}, hexokinase and glucose were added together with 10 mM $MgCl_2$. The presence of Ca^{2+} did not affect hexokinase action (see Section 2). After 30 s of the addition of hexokinase, the reaction was arrested. (B) shows the data in (A) plotted in the form of a second order rate equation as in reference [38]; the [γ-^{32}P]ATP binding rate constants (k_{+1}) were calculated from the slopes ($k_{+1} = m/RF_{1(0)} - ATP_{(0)}$). Calculated k_{+1} was $2.5 \times 10^5\,M^{-1}\,s^{-1}$ with Ca^{2+} (□), and $1.6 \times 10^5\,M^{-1}\,s^{-1}$ with Mg^{2+} (■).

hydrolysis that lasted until the totality of [γ-^{32}P]ATP was consumed i.e. approximately 1 min.

It has been reported that at substoichiometric ATP concentrations, F_1 may still work as under multisite conditions [34]. The sensitivity of ATP hydrolysis to azide has been used to distinguish between multisite and unisite hydrolysis i.e. the former, but not the latter, is inhibited by azide [35–37]. Therefore, to determine if the high ATPase activity observed with Ca^{2+} at substoichiometric ATP concentrations was indeed due to RF_1 working under unisite conditions, the effect of azide was determined at low and saturating [γ-^{32}P]ATP concentrations. For comparison, the experiments were also carried out in presence of Mg^{2+}.

With Ca^{2+}, azide inhibited hydrolysis at high ATP concentrations, but it did not affect hydrolysis when the concentration of ATP was lower than that of RF_1 (Fig. 2(A)). This indicates that with Ca^{2+} at substoichiometric ATP concentrations, only the high affinity catalytic site carried out hydrolysis. In presence of Mg^{2+}, azide inhibited hydrolysis at high ATP concentrations, but not at substoichiometric ATP concentrations. However, it was unexpectedly observed that azide enhanced unisite hydrolysis by about 50% (Fig. 2(B)). We have no explanation for these latter results.

3.1. Binding of [γ-^{32}P]ATP to R. rubrum F_1 incubated under unisite conditions with Mg^{2+} and Ca^{2+}

The finding that in presence of Ca^{2+}, RF_1 exhibits a higher rate of unisite ATP hydrolysis than with Mg^{2+} led us to study the effect that these divalent metal ions have on the various steps of the catalytic cycle. In a first approach, the binding of [γ-^{32}P]ATP to RF_1 was determined. RF_1 was incubated with Ca^{2+} or Mg^{2+} and substoichiometric concentrations of [γ-^{32}P]ATP; at various times, a large excess of hexokinase (+ glucose) was added to trap [γ-^{32}P]ATP that was not bound to RF_1. It should be noted that when Ca^{2+} was used, hexokinase was added together with Mg^{2+} in order to support hexokinase action. Controls in which [γ-^{32}P]ATP was added to a mixture that contained hexokinase, RF_1 and Mg^{2+}, with and without Ca^{2+}, showed that in the two conditions, 97% of [γ-^{32}P]ATP was transformed into glucose 6-[^{32}P]phosphate in less than 2 s.

With Mg^{2+} and Ca^{2+}, there was a progressive decrease in the amount of [γ-^{32}P]ATP that was accessible to hexokinase (Fig. 3(A)). With the two divalent metal ions, the time curves were markedly similar. From the amount of [γ-^{32}P]ATP that was accessible to hexokinase, the rate of [γ-^{32}P]ATP binding to RF_1 was calculated (Fig. 3(B)) according to references [30,38]. With Ca^{2+} and Mg^{2+} the rates were in the same range i.e. 2.5 and $1.6 \times 10^5\ M^{-1}\ s^{-1}$, respectively. Therefore, the large difference in the rate of unisite [γ-^{32}P]ATP hydrolysis with the two divalent metal ions is not due to differences in the rate of [γ-^{32}P]ATP binding.

3.2. Product release from R. rubrum F_1 undergoing unisite hydrolysis in presence of Mg^{2+} and Ca^{2+}

It has been shown in F_1 from various sources, that under unisite conditions, ATP at the catalytic site is continuously hydrolyzed and synthesized with an equilibrium constant that is near unity [19,21]. In RF_1

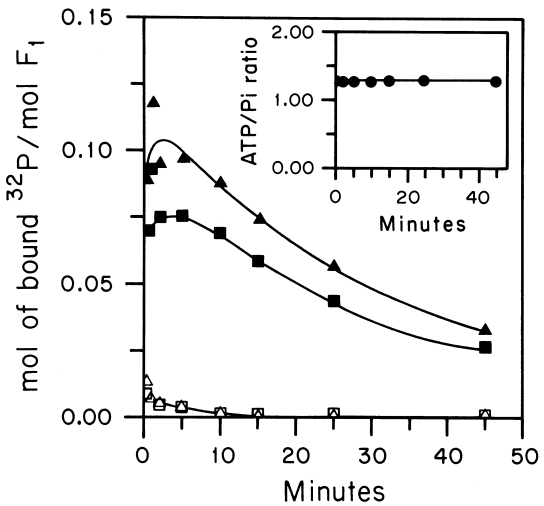

Fig. 4. Amount of [γ-^{32}P]ATP (▲,△) and [^{32}P]phosphate (■,□) bound to RF_1 under unisite conditions with Ca^{2+} (△,□) or Mg^{2+} (▲,■). At the times shown 100 μl of the reaction mixture were passed through Sephadex centrifugation–elution columns and received in a tube containing 100 μl of 12% TCA. An aliquot was used to determine total radioactivity (the sum of [γ-^{32}P]ATP and [^{32}P]P_i), and another aliquot was to determine [γ-^{32}P]ATP. The difference between total radioactivity and [γ-^{32}P]ATP was considered as [^{32}P]Pi bound to RF_1. The *inset* show the ratio of bound [γ-^{32}P]ATP:[^{32}P]phosphate in presence of Mg^{2+}. These values were calculated from the data (▲,■) in the main plot.

undergoing unisite hydrolysis of [γ-^{32}P]ATP, it was studied if Ca^{2+} and Mg^{2+} yield different ratios of [γ-^{32}P]ATP to [^{32}P]P_i at the catalytic site. In these experiments, RF_1 was exposed to unisite conditions, and at various times the reaction mixture was passed through Sephadex columns. In the eluates, the amount of protein, [γ-^{32}P]ATP and [^{32}P]P_i was determined. With Mg^{2+}, the amount of [γ-^{32}P]ATP and [^{32}P]P_i bound to RF_1 (Fig. 4), yielded an ATP:P_i ratio slightly higher than one (Fig. 4 inset). In presence of Ca^{2+} (Fig. 4), hardly any [γ-^{32}P]ATP or [^{32}P]P_i was detected in the eluates, indicating that the lifetime of [^{32}P]P_i at the catalytic site was shorter than with Mg^{2+}.

3.3. Effect of excess ATP on [γ-^{32}P]ATP bound to RF_1 under unisite conditions

It has been shown that the addition of excess ATP to F_1 undergoing unisite hydrolysis promotes a hydrolytic burst of the [γ-^{32}P]ATP bound under unisite conditions [20,32,39]. This results from the filling of empty catalytic sites which, through a cooperative reaction, produces a large increase in the rate of product release. This reaction was studied in RF_1 incubated with Mg^{2+} or Ca^{2+} (Fig. 5). RF_1 was incubated under unisite conditions and at various times, excess non-radioactive ATP was added. With Mg^{2+}, the results (Fig. 5(A)) were similar to those described in F_1 from other sources i.e. excess ATP produced a burst of hydrolysis of the previously bound [γ-^{32}P]ATP [20,39]. Nonetheless, it must be noted that excess ATP added in the first seconds of reaction time induced a relatively small enhancement of [γ-^{32}P]ATP hydrolysis. This is because at those times, not all [γ-^{32}P]ATP added had been bound to the enzyme (see Fig. 3).

With Ca^{2+}, notwithstanding the time of incubation under unisite conditions, the addition of excess ATP produced a small enhancement of [γ-^{32}P]ATP hydrolysis (Fig. 5(B)). This again indicates that with Ca^{2+}, [γ-^{32}P]ATP bound to the catalytic site is rapidly hydrolyzed, and thus there is hardly any bound [γ-^{32}P]ATP committed to hydrolysis.

The magnitude of the hydrolytic burst of [γ-^{32}P]ATP bound to F_1 under unisite conditions, has been used to determine the rate at which [γ-^{32}P]ATP binds to the catalytic site [19]. When the data of Fig.

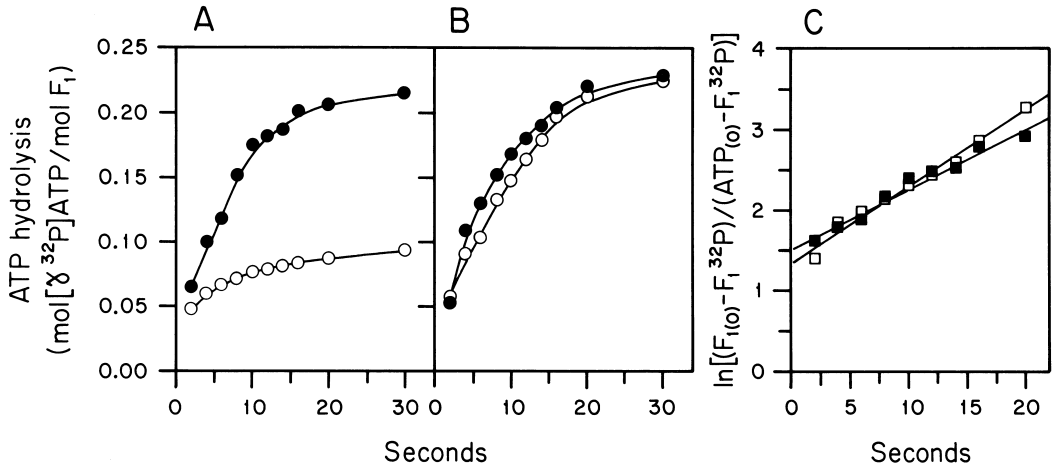

Fig. 5. Cold-chase experiments with RF_1 undergoing unisite [γ-^{32}P]ATP hydrolysis in the presence of Mg^{2+} (A) and Ca^{2+} (B). RF_1 was incubated with [γ-^{32}P]ATP (0.25 μM) under the standard conditions for unisite hydrolysis. At different reaction times, the samples were arrested with 300 μl of cold TCA (○), or alternatively 100 μl of non-radioactive ATP (4 mM) was added (●). With Mg^{2+} (A), the reaction was allowed to proceed for 1 min, or for 6 s with Ca^{2+} (B). At these times, the reactions were arrested and the amount of [γ-^{32}P]ATP hydrolyzed was determined (see Section 2, for details). The amount of cold ATP hydrolyzed was below 1% in presence of Mg^{2+} (A), and 3% in the samples with Ca^{2+} (B). Panel (C) is the graphical determination of k_{+1} using the data of cold-chase (●) in (A) and (B). The constants were calculated from the slopes using the equation described by Penefsky [30]: $k_{+1} = \text{slope}/F_{1(0)} \cdot ATP_{(0)}$, where $F_{1(0)}$ and $ATP_{(0)}$ are the initial concentrations of F_1 and [γ-^{32}P]ATP respectively. $F_1 \cdot {}^{32}P$ is the concentration of $F_1 \cdot {}^{32}P$ complex at the indicated times. The obtained constants were $1.26 \times 10^5\ M^{-1}\,s^{-1}$ and $9.8 \times 10^4\ M^{-1}\,s^{-1}$ for the conditions with Ca^{2+} (□) and Mg^{2+} (■), respectively.

5(A) and (B) were used to determine the rate of [γ-^{32}P]ATP binding (Fig. 5(C)), the values obtained (1.26×10^5 and $9.8 \times 10^4\ M^{-1}\ s^{-1}$ with Ca^{2+} and Mg^{2+}, respectively) were lower than those obtained with the hexokinase method ($2.5 \times 10^5\ M^{-1}\ s^{-1}$ with Ca^{2+}, and $1.6 \times 10^5\ M^{-1}\ s^{-1}$ with Mg^{2+}). It has been described [28] that the hydrolytic burst induced by excess ATP, is accompanied by release of a fraction of previously bound [γ-^{32}P]ATP. Therefore, it is possible that the value obtained by the cold-chase technique is underestimated.

4. Discussion

In chromatophores of *R. rubrum*, Mg^{2+}, but not Ca^{2+}, supports ADP phosphorylation driven by electron transport [17,40–42]. Also it has been shown that at saturating ATP concentrations, particulate RF_0F_1 exhibits a higher hydrolytic activity with Mg^{2+} than with Ca^{2+} [43]. Thus, the observation that soluble RF_1 shows multisite ATPase activity several-fold higher with Ca^{2+} than with Mg^{2+} [7] raises a number of questions concerning the mechanisms that operate during catalysis in particulate and soluble RF_1. In principle, the differences in hydrolytic rates of soluble RF_1 with Ca^{2+} and Mg^{2+} could reflect a difference in cooperativity among the three catalytic sites of RF_1, or alternatively, a difference in the kinetics of the events that occur at the catalytic sites. Our results show that the rate of unisite ATP hydrolysis by soluble RF_1 is several times higher with Ca^{2+}. Since under unisite conditions, only the high affinity catalytic site of a portion of the enzymes carries out ATP hydrolysis, the results indicate that independently of cooperativity, Ca^{2+} and Mg^{2+} affect differently the kinetics of ATP hydrolysis at the high affinity catalytic site of RF_1.

4.1. Mg^{2+}-dependent unisite [γ-^{32}P]ATP hydrolysis by RF_1

Most of the studies on unisite ATP hydrolysis by soluble F_1 from several sources have been made in presence of Mg^{2+} [2]. These results have shown that at substoichiometric concentrations of ATP to F_1, there is a rapid binding of [γ-^{32}P]ATP to the catalytic site of highest affinity [19]. At this site, the bound [γ-^{32}P]ATP is continuously hydrolyzed and resynthesized with an equilibrium constant that is near unity [19,20]. Indeed, if F_1 undergoing unisite [γ-^{32}P]ATP hydrolysis in presence of Mg^{2+} is filtered through Sephadex columns, the eluted enzyme contains [γ-^{32}P]ATP, as well as [^{32}P]P_i, the ratio between the two being close to one. Another relevant feature of unisite $Mg^{2+}-$ ATP hydrolysis is that the rate limiting step of the catalytic cycle is product release [19,21]. In fact, upon the addition of excess ATP to F_1 undergoing unisite [γ-^{32}P]ATP hydrolysis, the rate of hydrolysis of bound [γ-^{32}P]ATP increases several orders of magnitude. This results from the occupancy of other adenine nucleotide binding sites which in turn induce a 10^5-fold increase in the rate of product release [21].

In RF_1 incubated with Mg^{2+} and substoichiometric concentrations of [γ-^{32}P]ATP, there is a rapid binding of [γ-^{32}P]ATP to RF_1, and it was observed that in RF_1 undergoing unisite [γ-^{32}P]ATP hydrolysis, [^{32}P]P_i derived from [γ-^{32}P]ATP hydrolysis remains bound to RF_1 after passage through centrifuge columns. The ratio of [γ-^{32}P]ATP:[^{32}P]P_i at the catalytic site was 1.2 (see Fig. 4 inset). Likewise, the addition of high ATP concentrations to RF_1 undergoing unisite [γ-^{32}P]ATP hydrolysis produces a hydrolytic burst of the previously bound [γ-^{32}P]ATP. This set of observations indicates that in presence of Mg^{2+}. RF_1 does not behave differently from F_1 from other sources.

4.2. Ca^{2+}-dependent unisite [γ-^{32}P]ATP hydrolysis by RF_1

Our data show that with Ca^{2+}, unisite hydrolysis is significantly higher than with Mg^{2+}, albeit the binding of [γ-^{32}P]ATP to RF_1 with both divalent cations is in the same time range. It was also found that the passage of RF_1 undergoing unisite hydrolysis of [γ-^{32}P]ATP in presence of Ca^{2+} through Sephadex columns yielded an enzyme that did not contain [^{32}P]P_i. Therefore, it would appear that in comparison to Mg^{2+}, the high rate of unisite [γ-^{32}P]ATP hydrolysis that is observed with Ca^{2+} is consequence of a high rate of product release from the catalytic site. This in turn suggests that the affinity of the catalytic site for phosphate with Ca^{2+} is lower than with Mg^{2+}. Thermodynamics analysis of the catalytic cy-

Table 1
Rate promotion from the unisite to multisite catalysis in RF_1

Unisite hydrolysis (s^{-1})	Multisite hydrolysis (s^{-1}) [a]	Rate promotion (fold) [b]
6.8×10^{-5} [c]	0.54	8×10^3 Mg^{2+}-ATPase
3.7×10^{-3} [d]	48	1.3×10^4 Ca^{2+}-ATPase

[a] Multisite turnover rates were calculated from linear rates of P_i formation.
[b] Rate promotion is expressed as the ratio of multisite turnover to unisite turnover.
[c] Mg^{2+}-dependent unisite turnover was calculated from the slope of [γ-^{32}P]ATP hydrolysis between 14 s to 20 min. At 14 s all [γ-^{32}P]ATP added had bound to RF_1 (see Fig. 3).
[d] The turnover in the unisite Ca^{2+}-ATPase activity was calculated in the slope between 14–30 s.

cle of ATP synthesis shows that phosphate binding is a major energy requiring step [44]. Therefore, it is possible that with Ca^{2+}, energy requirements for phosphate binding are exceedingly high, and in consequence, light-driven ATP synthesis cannot take place.

In RF_1 ATP hydrolysis with Ca^{2+} is faster than with Mg^{2+}. However, this does not necessarily imply that in the presence of Ca^{2+}, the rate limiting step shifts to another step of the catalytic cycle. In this context, it is instructive to compare the rates of unisite and multisite by RF_1 with Ca^{2+} and Mg^{2+}. As shown in Table 1 the ratio of multisite:unisite hydrolysis was of similar extent with the two divalent cations. Thus, it appears that the rates of product release vary in parallel with Ca^{2+} and Mg^{2+}. Acceleration of [^{32}P]phosphate release by the occupancy of other sites in chloroplast F_1 in presence of Ca^{2+} has been reported [45,46].

Miwa and Yoshida [47] and Yokoyama et al. [48] showed that in F_1–$\alpha_3\beta_3$ complexes that lack γ-subunit, the ATPase activity is two-fold higher with Ca^{2+} than with Mg^{2+}; this suggested that interactions of the catalytic subunits with the γ-subunit are not necessarily involved in the expression of high catalytic rates with Ca^{2+}. The latter reports are in consonance with the present data, since our overall results indicate that Ca^{2+} bears directly on the events and/or molecular rearrangements that occur at the high affinity catalytic site of RF_1. However, it is noted that in V_c-ATPase, the interactions between subunits affect strongly the response to Ca^{2+} and Mg^{2+} [49].

In regard to the action of Ca^{2+} and Mg^{2+} on the retention time of the products of unisite ATP hydrolysis, knowledge of the structure of the catalytic site loaded with Mg^{2+}-ADP or Ca^{2+}-ADP would be valuable, but these data are not available. However, the crystal structure of F_1 with one of its sites occupied by Mg^{2+}-ADP (β_{DP} subunit, in the terminology of Abrahams et al. [50]), and that of dethiobiotin synthetase in complex with Ca^{2+}-ADP show that Ca^{2+} and Mg^{2+} are coordinated differently at the active sites [51]. Moreover in the crystal structure of the ATPase fragment of the 70 kDa heat shock protein, Flaherty et al. [52] observed that the active site occupied by AMP–PNP, Ca^{2+} and Mg^{2+} bind to locus 2.3 Å apart. Therefore, these findings suggest that the rate of ADP release may be related to differences in the coordination of Ca^{2+} and Mg^{2+} at the catalytic site.

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