

## Unisite ATP hydrolysis by soluble *Rhodospirillum rubrum* F<sub>1</sub>–ATPase is accelerated by Ca<sup>2+</sup>

Ernesto Maldonado <sup>a</sup>, Georges Dreyfus <sup>a,\*</sup>, José J. García <sup>a,1</sup>, Armando Gómez-Puyou <sup>b</sup>,  
Marietta Tuena de Gómez-Puyou <sup>a</sup>

<sup>a</sup> Departamento de Genética Molecular, Universidad Nacional Autónoma de México, Apartado Postal 70-243,  
Mexico D.F. 04510, Mexico

<sup>b</sup> 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<sub>1</sub> from the *Rhodospirillum rubrum* (RF<sub>1</sub>) exhibits a higher rate of hydrolysis with Ca<sup>2+</sup> than with Mg<sup>2+</sup>. The mechanisms involved in the expression of a higher catalytic activity with Ca<sup>2+</sup> were explored by measuring the ATPase activity of RF<sub>1</sub> at substoichiometric concentrations of ATP (unisite conditions). At a ratio of 0.25 [γ-<sup>32</sup>P]ATP per RF<sub>1</sub>, the enzyme exhibited a 50 times higher hydrolytic rate with Ca<sup>2+</sup> than with Mg<sup>2+</sup>. The rate of [γ-<sup>32</sup>P]ATP binding to RF<sub>1</sub> was in the same range with the two divalent metal ions. Centrifugation–filtration of RF<sub>1</sub> exposed to substoichiometric [γ-<sup>32</sup>P]ATP concentrations and Mg<sup>2+</sup> through Sephadex columns yielded an enzyme that contained [γ-<sup>32</sup>P]ATP and [<sup>32</sup>P]phosphate in a stoichiometry that was close to one. In the presence of Ca<sup>2+</sup>, the eluted enzyme did not contain [γ-<sup>32</sup>P]ATP nor [<sup>32</sup>P]phosphate. This indicated that the rate of product release was faster with Ca<sup>2+</sup> than with Mg<sup>2+</sup>. 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<sub>1</sub> in presence of Ca<sup>2+</sup> strongly suggests that the retention time of products is decreased in the presence of this cation. © 1998 Elsevier Science B.V.

**Keywords:** ATPase, F<sub>1</sub>–; Unisite ATP hydrolysis; Ca<sup>2+</sup>; Mg<sup>2+</sup>; Product release; (*Rhodospirillum rubrum*)

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### 1. Introduction

The ATP synthase of energy transducing membranes catalyzes the synthesis of ATP from ADP and

P<sub>i</sub> (for reviews, see [1,2]). The energy for the synthesis of ATP is provided by electrochemical H<sup>+</sup> gradients derived from electron transport that is initiated by substrate oxidation, or by light in photosynthetic organisms. The ATP synthase of the photosynthetic bacterium *Rhodospirillum 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<sub>0</sub>) whose function is to channel H<sup>+</sup> to a portion of the ATP synthase known

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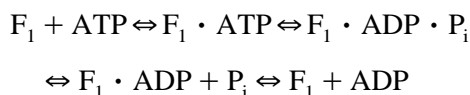
\* Corresponding author. Fax: +52 5 616 2282; E-mail: gdreyfus@ifcsun1.ifisiol.unam.mx

<sup>1</sup> Present address: Instituto Nacional de Cardiología “Ignacio Chávez”, Departamento de Bioquímica, Juan Badiano 1, Mexico D.F. 14080, Mexico.

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 subtilis* [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



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  $[\gamma\text{-}^{32}P]ATP$  according to reference [23]; its specific activity was  $2\text{--}3 \times 10^6$  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  $-70^\circ\text{C}$ . 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 $[\gamma\text{-}^{32}P]ATP$ hydrolysis and cold-chase experiments

In the standard method for measuring unisite  $[\gamma\text{-}^{32}P]ATP$  hydrolysis, 50  $\mu\text{l}$  of  $RF_1$  at a concentration of 2  $\mu\text{M}$  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  $\mu\text{l}$  of 0.5  $\mu\text{M}$  of  $[\gamma\text{-}^{32}P]ATP$ . At the desired times, the reaction was arrested with 300  $\mu\text{l}$  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  $[\gamma\text{-}^{32}P]ATP$

that remained in the water phase was used to calculate the amount of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  hydrolyzed. In cold-chase experiments [19], 100  $\mu\text{l}$  of non-radioactive  $\text{Ca}^{2+}$ - or  $\text{Mg}^{2+}$ -ATP (4 mM final concentration) were added to  $\text{RF}_1$  undergoing unisite  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  hydrolysis. When  $\text{Ca}^{2+}$  was used, the reaction was allowed to proceed for 6 s; with  $\text{Mg}^{2+}$  the reaction was arrested after 1 min. The amount of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  that was not hydrolyzed was determined as described above.

### 2.3. Determination of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ bound to $\text{RF}_1$ by the hexokinase-glucose trap method

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$  binding to  $\text{RF}_1$  under unisite conditions was determined by the hexokinase-glucose trap method [29]. For these experiments,  $\text{RF}_1$  was incubated with  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  in the standard mixture for unisite  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  hydrolysis, except that the final volume was 40  $\mu\text{l}$ . At the desired times, the samples were supplemented with 300  $\mu\text{l}$  of a mixture at pH 8.0 that contained 20 units of hexokinase, 10 mM glucose and 10 mM  $\text{MgCl}_2$ ; the reactions were arrested 30 s later with HCl (1.3 N, final concentration). The activity of hexokinase added was around  $5 \times 10^3$ -folds higher than the hydrolytic activity of  $\text{RF}_1$  at saturating ATP concentrations. To determine the amount of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  that had been converted into

glucose 6- $[\text{}^{32}\text{P}]\text{phosphate}$ , the samples were placed in boiling water (92°C) for 30 min. Subsequently,  $[\text{}^{32}\text{P}]\text{P}_i$  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- $[\text{}^{32}\text{P}]\text{phosphate}$  formed. From the data, the rate of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  binding was calculated according to reference [30]. Prior to the experiments two assays were carried out: (i) In the experiments with  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  had to be added with hexokinase in order to support hexokinase action; experiments in the absence of  $\text{RF}_1$  showed that under these conditions, hexokinase converted around 97% of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  into glucose 6- $[\text{}^{32}\text{P}]\text{phosphate}$  in less than 2 s; (ii) the effect of heat treatment on  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  and glucose 6- $[\text{}^{32}\text{P}]\text{phosphate}$  was determined; it was observed that heat treatment produced cleavage of more than 97% of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ ; and that on the average 98% of glucose 6- $[\text{}^{32}\text{P}]\text{phosphate}$  was heat resistant.

### 2.4. Determination of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and $[\text{}^{32}\text{P}]\text{phosphate}$ bound to $\text{RF}_1$ by the centrifugation–filtration method

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

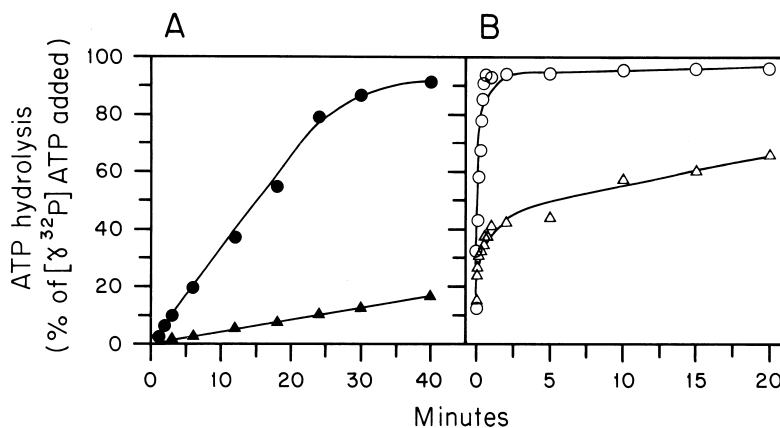


Fig. 1. Time course ATP hydrolysis by  $\text{RF}_1$  at saturating (A) and substoichiometric (B) ATP concentrations. In (A), the reaction was carried out with 3 mM ATP and 3 mM of  $\text{Mg}^{2+}$  ( $\blacktriangle$ ) or  $\text{Ca}^{2+}$  ( $\bullet$ ); the reaction was initiated by adding 12  $\mu\text{g}$  ( $\blacktriangle$ ) or 2  $\mu\text{g}$  ( $\bullet$ )  $\text{RF}_1$  to the reaction medium (final volume = 100  $\mu\text{l}$ ) as indicated under Section 2. At the times indicated, the reactions were arrested and the amount of  $\text{P}_i$  formed was determined. Unisite ATPase activity (B) was started by mixing of equal volumes of  $\text{RF}_1$  (2  $\mu\text{M}$ ) and  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  (0.5  $\mu\text{M}$ ) in the unisite standard buffer, in the presence of 2 mM of  $\text{Mg}^{2+}$  ( $\triangle$ ) or  $\text{Ca}^{2+}$  ( $\circ$ ); at the times shown the reactions were arrested with TCA, and the amount of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  that remained was determined.

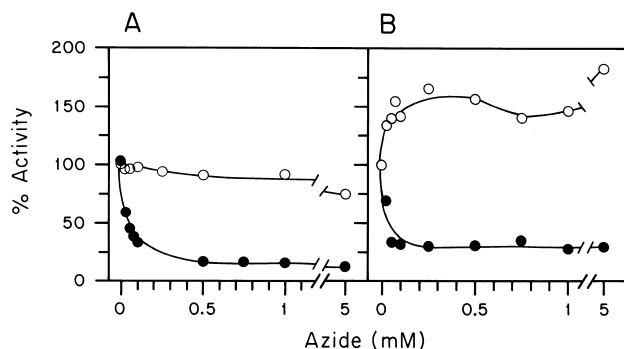


Fig. 2. Effect of different concentrations of azide on ATP hydrolysis by  $\text{RF}_1$  under multisite (●) and unisite (○) conditions in presence of  $\text{Ca}^{2+}$  (A) or  $\text{Mg}^{2+}$  (B). The experimental mixtures were as in Fig. 1 except that azide was added at the indicated concentrations. Under unisite conditions, (○) represent the fraction of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  hydrolyzed after 8 s of incubation with  $\text{Ca}^{2+}$  (panel A) or after 5 min of incubation with  $\text{Mg}^{2+}$  (panel B). In the absence of azide, the fractions of hydrolyzed  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ , were 0.18 and 0.13 mol  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ /mol  $\text{RF}_1$  with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , respectively. Under multisite conditions (●), without azide, the ATPase activity (100%) was 6 and 0.1  $\mu\text{mol}/\text{min}/\text{mg}$  with  $\text{Ca}^{2+}$ , and  $\text{Mg}^{2+}$ , respectively.

cal sample was extracted twice with butyl acetate after formation of the phosphomolybdate complex. The radioactivity in the aqueous phase was considered as  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  bound to  $\text{RF}_1$ . The difference between total radioactivity and  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  was equal to  $[\text{P}_i]$  bound to  $\text{RF}_1$ . Protein was determined,

according to Smith et al. [31], in eluates of identical samples, except that these were received in 100  $\mu\text{l}$  of 10% SDS.

### 3. Results

The ATPase activity of  $\text{RF}_1$  was measured at saturating and substoichiometric concentrations of ATP (Fig. 1(A) and (B), respectively) in presence of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . In confirmation of previous data [7] ATP hydrolysis was higher with  $\text{Ca}^{2+}$  under multisite conditions. Under unisite conditions in presence of  $\text{Mg}^{2+}$ , there was a rapid initial burst of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  hydrolysis that was followed by a relatively slow phase of hydrolysis that continued until  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  was exhausted (Fig. 1(B)). This pattern has been observed with  $\text{F}_1$  from various sources [19,20,32]. With  $\text{Mg}^{2+}$  the rapid phase reflects binding and splitting of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  at the high affinity catalytic site, where an equilibrium between  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  and ADP and  $[\text{P}_i]$  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  $\text{RF}_1$  in the presence of  $\text{Ca}^{2+}$  differed markedly from that observed with  $\text{Mg}^{2+}$  (Fig. 1(B)). With  $\text{Ca}^{2+}$ , there was a continuous relatively rapid rate of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$

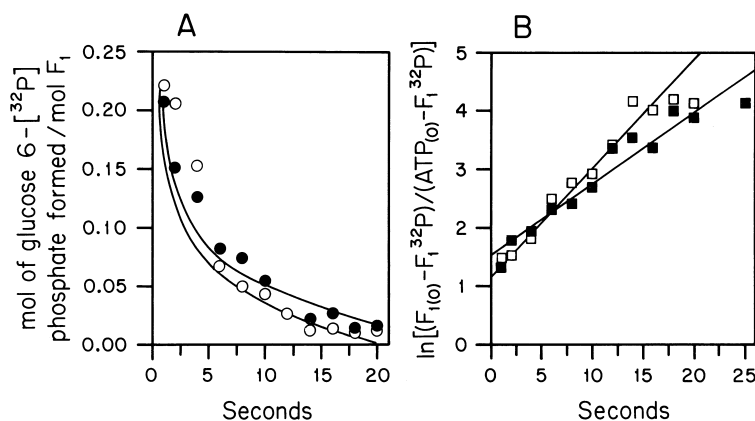


Fig. 3. Accessibility of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  to hexokinase in  $\text{RF}_1$  incubated under unisite conditions. In (A),  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  was incubated under unisite conditions with  $\text{Mg}^{2+}$  (●) or  $\text{Ca}^{2+}$  (○). At the times shown, hexokinase (20 units) and 10 mM glucose was added. In the tubes incubated with  $\text{Ca}^{2+}$ , hexokinase and glucose were added together with 10 mM  $\text{MgCl}_2$ . The presence of  $\text{Ca}^{2+}$  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  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  binding rate constants ( $k_{+1}$ ) were calculated from the slopes ( $k_{+1} = m / (F_{1(0)} - ATP_{(0)})$ ). Calculated  $k_{+1}$  was  $2.5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$  with  $\text{Ca}^{2+}$  (□), and  $1.6 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$  with  $\text{Mg}^{2+}$  (■).

hydrolysis that lasted until the totality of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  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  $\text{Ca}^{2+}$  at substoichiometric ATP concentrations was indeed due to  $\text{RF}_1$  working under unisite conditions, the effect of azide was determined at low and saturating  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  concentrations. For comparison, the experiments were also carried out in presence of  $\text{Mg}^{2+}$ .

With  $\text{Ca}^{2+}$ , azide inhibited hydrolysis at high ATP concentrations, but it did not affect hydrolysis when the concentration of ATP was lower than that of  $\text{RF}_1$  (Fig. 2(A)). This indicates that with  $\text{Ca}^{2+}$  at substoichiometric ATP concentrations, only the high affinity catalytic site carried out hydrolysis. In presence of  $\text{Mg}^{2+}$ , 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 $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ to *R. rubrum* $F_1$ incubated under unisite conditions with $\text{Mg}^{2+}$ and $\text{Ca}^{2+}$

The finding that in presence of  $\text{Ca}^{2+}$ ,  $\text{RF}_1$  exhibits a higher rate of unisite ATP hydrolysis than with  $\text{Mg}^{2+}$  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  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  to  $\text{RF}_1$  was determined.  $\text{RF}_1$  was incubated with  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  and substoichiometric concentrations of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ ; at various times, a large excess of hexokinase (+ glucose) was added to trap  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  that was not bound to  $\text{RF}_1$ . It should be noted that when  $\text{Ca}^{2+}$  was used, hexokinase was added together with  $\text{Mg}^{2+}$  in order to support hexokinase action. Controls in which  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  was added to a mixture that contained hexokinase,  $\text{RF}_1$  and  $\text{Mg}^{2+}$ , with and without  $\text{Ca}^{2+}$ , showed that in the two conditions, 97% of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  was transformed into glucose 6- $[\text{P}]$ phosphate in less than 2 s.

With  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$ , there was a progressive decrease in the amount of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  that was accessible to hexokinase (Fig. 3(A)). With the two divalent metal ions, the time curves were markedly similar. From the amount of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  that was accessible to hexokinase, the rate of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  binding to  $\text{RF}_1$  was calculated (Fig. 3(B)) according to references [30,38]. With  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  the rates were in the same range i.e.  $2.5$  and  $1.6 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ , respectively. Therefore, the large difference in the rate of unisite  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  hydrolysis with the two divalent metal ions is not due to differences in the rate of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  binding.

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

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  $\text{RF}_1$

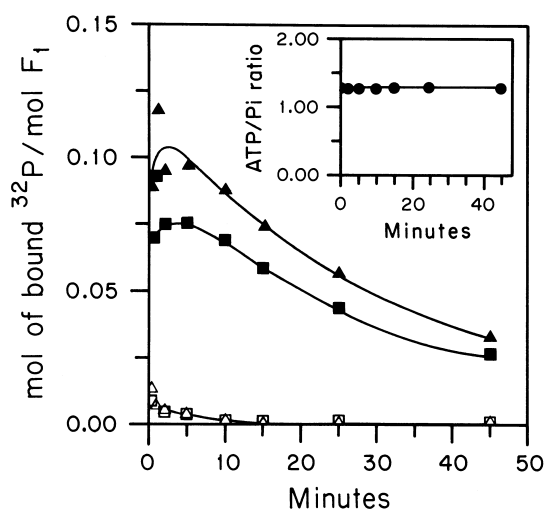


Fig. 4. Amount of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  ( $\blacktriangle, \triangle$ ) and  $[\text{P}]^{32}\text{phosphate}$  ( $\blacksquare, \square$ ) bound to  $\text{RF}_1$  under unisite conditions with  $\text{Ca}^{2+}$  ( $\triangle, \square$ ) or  $\text{Mg}^{2+}$  ( $\blacktriangle, \blacksquare$ ). At the times shown  $100 \mu\text{l}$  of the reaction mixture were passed through Sephadex centrifugation–elution columns and received in a tube containing  $100 \mu\text{l}$  of 12% TCA. An aliquot was used to determine total radioactivity (the sum of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  and  $[\text{P}]^{32}\text{P}_i$ ), and another aliquot was to determine  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ . The difference between total radioactivity and  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  was considered as  $[\text{P}]^{32}\text{P}_i$  bound to  $\text{RF}_1$ . The inset shows the ratio of bound  $[\gamma\text{-}^{32}\text{P}]\text{ATP}:[\text{P}]^{32}\text{phosphate}$  in presence of  $\text{Mg}^{2+}$ . These values were calculated from the data ( $\blacktriangle, \blacksquare$ ) in the main plot.

undergoing unisite hydrolysis of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ , it was studied if  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  yield different ratios of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  to  $[\text{P}]\text{P}_i$  at the catalytic site. In these experiments,  $\text{RF}_1$  was exposed to unisite conditions, and at various times the reaction mixture was passed through Sephadex columns. In the eluates, the amount of protein,  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  and  $[\text{P}]\text{P}_i$  was determined. With  $\text{Mg}^{2+}$ , the amount of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  and  $[\text{P}]\text{P}_i$  bound to  $\text{RF}_1$  (Fig. 4), yielded an ATP: $\text{P}_i$  ratio slightly higher than one (Fig. 4 inset). In presence of  $\text{Ca}^{2+}$  (Fig. 4), hardly any  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  or  $[\text{P}]\text{P}_i$  was detected in the eluates, indicating that the lifetime of  $[\text{P}]\text{P}_i$  at the catalytic site was shorter than with  $\text{Mg}^{2+}$ .

### 3.3. Effect of excess ATP on $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ bound to $\text{RF}_1$ under unisite conditions

It has been shown that the addition of excess ATP to  $\text{F}_1$  undergoing unisite hydrolysis promotes a hydrolytic burst of the  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  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  $\text{RF}_1$  incubated with  $\text{Mg}^{2+}$  or  $\text{Ca}^{2+}$  (Fig. 5).  $\text{RF}_1$  was incubated under unisite conditions and at various times, excess non-radioactive ATP was added. With  $\text{Mg}^{2+}$ , the results (Fig. 5(A)) were similar to those described in  $\text{F}_1$  from other sources i.e. excess ATP produced a burst of hydrolysis of the previously bound  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  [20,39]. Nonetheless, it must be noted that excess ATP added in the first seconds of reaction time induced a relatively small enhancement of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  hydrolysis. This is because at those times, not all  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  added had been bound to the enzyme (see Fig. 3).

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

The magnitude of the hydrolytic burst of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  bound to  $\text{F}_1$  under unisite conditions, has been used to determine the rate at which  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  binds to the catalytic site [19]. When the data of Fig.

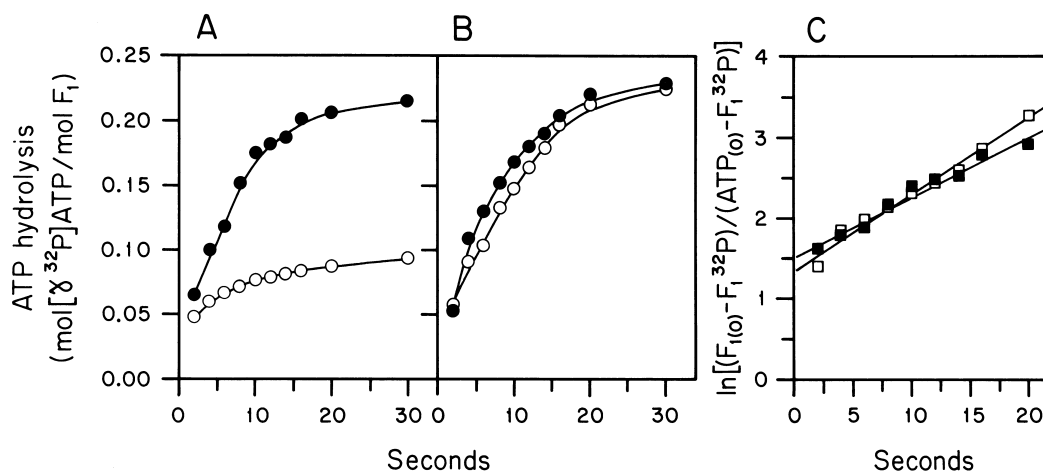


Fig. 5. Cold-chase experiments with  $\text{RF}_1$  undergoing unisite  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  hydrolysis in the presence of  $\text{Mg}^{2+}$  (A) and  $\text{Ca}^{2+}$  (B).  $\text{RF}_1$  was incubated with  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  ( $0.25\ \mu\text{M}$ ) under the standard conditions for unisite hydrolysis. At different reaction times, the samples were arrested with  $300\ \mu\text{l}$  of cold TCA (○), or alternatively  $100\ \mu\text{l}$  of non-radioactive ATP ( $4\ \text{mM}$ ) was added (●). With  $\text{Mg}^{2+}$  (A), the reaction was allowed to proceed for 1 min, or for 6 s with  $\text{Ca}^{2+}$  (B). At these times, the reactions were arrested and the amount of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  hydrolyzed was determined (see Section 2, for details). The amount of cold ATP hydrolyzed was below 1% in presence of  $\text{Mg}^{2+}$  (A), and 3% in the samples with  $\text{Ca}^{2+}$  (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]:  $k_{+1} = \text{slope} / F_{1(0)} \cdot \text{ATP}_{(0)}$ , where  $F_{1(0)}$  and  $\text{ATP}_{(0)}$  are the initial concentrations of  $\text{F}_1$  and  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  respectively.  $F_1^{32P}$  is the concentration of  $\text{F}_1 \cdot ^{32P}$  complex at the indicated times. The obtained constants were  $1.26 \times 10^5\ \text{M}^{-1}\text{s}^{-1}$  and  $9.8 \times 10^4\ \text{M}^{-1}\text{s}^{-1}$  for the conditions with  $\text{Ca}^{2+}$  (□) and  $\text{Mg}^{2+}$  (■), respectively.

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

#### 4. Discussion

In chromatophores of *R. rubrum*,  $\text{Mg}^{2+}$ , but not  $\text{Ca}^{2+}$ , supports ADP phosphorylation driven by electron transport [17,40–42]. Also it has been shown that at saturating ATP concentrations, particulate  $\text{RF}_0\text{F}_1$  exhibits a higher hydrolytic activity with  $\text{Mg}^{2+}$  than with  $\text{Ca}^{2+}$  [43]. Thus, the observation that soluble  $\text{RF}_1$  shows multisite ATPase activity several-fold higher with  $\text{Ca}^{2+}$  than with  $\text{Mg}^{2+}$  [7] raises a number of questions concerning the mechanisms that operate during catalysis in particulate and soluble  $\text{RF}_1$ . In principle, the differences in hydrolytic rates of soluble  $\text{RF}_1$  with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  could reflect a difference in cooperativity among the three catalytic sites of  $\text{RF}_1$ , 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  $\text{RF}_1$  is several times higher with  $\text{Ca}^{2+}$ . 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,  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  affect differently the kinetics of ATP hydrolysis at the high affinity catalytic site of  $\text{RF}_1$ .

##### 4.1. $\text{Mg}^{2+}$ -dependent unisite $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ hydrolysis by $\text{RF}_1$

Most of the studies on unisite ATP hydrolysis by soluble  $\text{F}_1$  from several sources have been made in presence of  $\text{Mg}^{2+}$  [2]. These results have shown that at substoichiometric concentrations of ATP to  $\text{F}_1$ , there is a rapid binding of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  to the catalytic site of highest affinity [19]. At this site, the bound

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$  is continuously hydrolyzed and re-synthesized with an equilibrium constant that is near unity [19,20]. Indeed, if  $\text{F}_1$  undergoing unisite  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  hydrolysis in presence of  $\text{Mg}^{2+}$  is filtered through Sephadex columns, the eluted enzyme contains  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ , as well as  $[\text{P}^{32}]\text{P}_i$ , the ratio between the two being close to one. Another relevant feature of unisite  $\text{Mg}^{2+}$ –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  $\text{F}_1$  undergoing unisite  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  hydrolysis, the rate of hydrolysis of bound  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  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  $\text{RF}_1$  incubated with  $\text{Mg}^{2+}$  and substoichiometric concentrations of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ , there is a rapid binding of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  to  $\text{RF}_1$ , and it was observed that in  $\text{RF}_1$  undergoing unisite  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  hydrolysis,  $[\text{P}^{32}]\text{P}_i$  derived from  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  hydrolysis remains bound to  $\text{RF}_1$  after passage through centrifuge columns. The ratio of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}:[\text{P}^{32}]\text{P}_i$  at the catalytic site was 1.2 (see Fig. 4 inset). Likewise, the addition of high ATP concentrations to  $\text{RF}_1$  undergoing unisite  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  hydrolysis produces a hydrolytic burst of the previously bound  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ . This set of observations indicates that in presence of  $\text{Mg}^{2+}$ ,  $\text{RF}_1$  does not behave differently from  $\text{F}_1$  from other sources.

##### 4.2. $\text{Ca}^{2+}$ -dependent unisite $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ hydrolysis by $\text{RF}_1$

Our data show that with  $\text{Ca}^{2+}$ , unisite hydrolysis is significantly higher than with  $\text{Mg}^{2+}$ , albeit the binding of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  to  $\text{RF}_1$  with both divalent cations is in the same time range. It was also found that the passage of  $\text{RF}_1$  undergoing unisite hydrolysis of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  in presence of  $\text{Ca}^{2+}$  through Sephadex columns yielded an enzyme that did not contain  $[\text{P}^{32}]\text{P}_i$ . Therefore, it would appear that in comparison to  $\text{Mg}^{2+}$ , the high rate of unisite  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  hydrolysis that is observed with  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  is lower than with  $\text{Mg}^{2+}$ . 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 ( $s^{-1}$ ) <sup>a</sup>	Rate promotion (fold) <sup>b</sup>
$6.8 \times 10^{-5}$ <sup>c</sup>	0.54	$8 \times 10^3$ $Mg^{2+}$ -ATPase
$3.7 \times 10^{-3}$ <sup>d</sup>	48	$1.3 \times 10^4$ $Ca^{2+}$ -ATPase

<sup>a</sup> Multisite turnover rates were calculated from linear rates of  $P_i$  formation.<sup>b</sup> Rate promotion is expressed as the ratio of multisite turnover to unisite turnover.<sup>c</sup>  $Mg^{2+}$ -dependent unisite turnover was calculated from the slope of  $[\gamma\text{-}^{32}P]ATP$  hydrolysis between 14 s to 20 min. At 14 s all  $[\gamma\text{-}^{32}P]ATP$  added had bound to  $RF_1$  (see Fig. 3).<sup>d</sup> 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  $[\text{}^{32}P]$ 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  $F_1\text{-}\alpha_3\beta_3$  complexes that lack  $\gamma$ -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  $\gamma$ -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 ( $\beta_{DP}$  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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