Demonstration of two binding sites for ADP on the isolated β-subunit of the *Rhodospirillum rubrum* $R_1F_0F_1$-ATP synthase

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Two ADP binding sites have been demonstrated on the reconstitutively active β-subunit, that was removed from the *Rhodospirillum rubrum* membrane-bound ATP synthase. One is a high affinity site ($K_d = 0.7$ μM) that does not require $MgCl_2$ and is unaffected by it. The second is a low affinity binding site ($K_d = 80$ μM) that is absolutely dependent on $MgCl_2$. For stable binding of ADP to this site, $MgCl_2$ must be present not only during the binding step but also during the elution-centrifugation step used to separate the β-subunit bound [^{3}H]ADP from the free ligand. When $MgCl_2$ is removed together with the free ligand [^{3}H]ADP dissociates very rapidly from this second binding site.

Rhodospirillum rubrum *F_0F_1-ATP synthase* *β-Subunit* *ADP-binding site* *$MgCl_2$ dependence* *Binding affinity*

1. INTRODUCTION

The characterization of substrate binding sites on the proton-translocating F_0F_1-ATP synthase is a prerequisite for the elucidation of its mechanism of action. Previous studies have shown that this enzyme complex has several nucleotide binding sites that reside in the two larger subunits, α and β [1–4]. They seem to include both catalytic and regulatory sites, but the exact subunit location of each category is not clear.

Individual α- and β-subunits that were isolated in an active form, have also been shown to contain nucleotide binding sites [5–9]. The most promising approach to the identification of the number, and characterization of the role of these sites is to examine the direct binding of labeled ADP and ATP to the isolated, active α- and β-subunits. Such studies have been carried out until now only on the α-subunit of *Escherichia coli* [5] and on the β-subunit of *R. rubrum* [8]. These studies have identified one, high affinity, nucleotide binding site on the α-subunit, whereas on the β-subunit two nucleotide binding sites have been found. One of them, which is very similar to the binding site located on the α-subunit, is a high affinity site, that is independent on $MgCl_2$ and binds either ATP or ADP. The second is a low affinity site that is absolutely dependent on $MgCl_2$ and has been shown to bind only ATP [8]. Recently, we have also identified one, low affinity, $MgCl_2$-dependent P_i binding site on the *R. rubrum* β-subunit [10]. It seems to be located at the low affinity ATP binding site and its properties suggest it is a catalytic site.

In light of these results the failure to observe a low affinity binding site for ADP on the isolated β-subunit [8] was rather surprising; the more so, since we have earlier reported that this β-subunit does rebind to β-less chromatophores and restores fully their ATP synthesis as well as hydrolysis activities [11,12]. The present study was initiated to determine if by changing the conditions of the binding assay a low affinity binding site for ADP could be demonstrated on the isolated β-subunit.

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2. EXPERIMENTAL

Growth of *R. rubrum* cells, preparation of chromatophores and isolation and purification of the reconstitutively active β-subunit have been earlier described [8,11,12]. In the experiments reported here an electrophoretically pure β-subunit, which restored 92–97% of the photophosphorylation or Mg^{2+}-ATPase activities of β-less chromatophores, was used. Before incubation of the β-subunit with [^{3}H]ADP, a concentrated solution of EDTA was added to β at 2–3 mg/ml in the storage buffer (50 mM Tricine–NaOH, pH 8.0; 4 mM ATP; 4 mM $MgCl_2$ and 10% glycerol) so that the final concentration of EDTA was 2 mM. The β-subunit was then freed from the storage buffer by elution-centrifugation [13] in Sephadex G-50 columns equilibrated with 50 mM Tricine–NaOH, pH 8.0; 20% glycerol; 2 mM EDTA and 50 mM NaCl. The elution-centrifugation step was repeated twice. After this treatment <0.1 mol ATP remained bound to the β-subunit [10], but it retained its reconstitutive activity and its nucleotide binding capacity when kept for at least several hours at room temperature (23°C).

[2,8-^{3}H]ADP (26–30 Ci/mmol) was obtained from New England Nuclear and was diluted with chromatographically purified non-radioactive ADP to give a specific radioactivity of 1.4×10^5 cpm/nmol.

Binding studies were carried out by incubating [^{3}H]ADP with the β-subunit at 10 μM [8] in TG buffer (50 mM Tricine–NaOH, pH 8.0; 20% glycerol) containing either varying concentrations of $MgCl_2$, as indicated in each figure, or 2 mM EDTA in the absence of $MgCl_2$. The incubation was initiated by addition of the β-subunit, and after 1 h at 23°C the mixture was subjected to elution-centrifugation on Sephadex columns to separate the free and bound [^{3}H]ADP, as in [8]. The columns were preequilibrated with the buffer indicated in each figure. The effluent from each column was diluted with 1 ml water and appropriate aliquots were assayed for ^{3}H radioactivity and protein content.

Protein was determined as in [14]. ^{3}H radioactivity was measured by liquid scintillation counting [13]. Binding data were calculated by using an M_r of 50000 for the β-subunit [15]. The dissociation constants were obtained by treatment of Scatchard plots as in [16].

3. RESULTS AND DISCUSSION

The reconstitutively active β-subunit isolated from the *R. rubrum* membrane-bound ATP synthase has been found to bind 2 mol ATP, but only 1 mol ADP/mol, when examined by the elution-centrifugation technique [8]. This technique was shown to be very effective in removing unbound ions from a protein sample [13]. So it can be used to separate bound and free nucleotides, provided that the bound nucleotide is stable and does not dissociate from its binding site during centrifugation on the Sephadex column. If, however, nucleotide binding requires the presence of an additional effector, i.e., $MgCl_2$ for ATP binding to the second site on β [8], the bound nucleotide might dissociate when this effector is removed, together with the free nucleotide, during the centrifugation step. Our results indicate that this was not the case with the $MgCl_2$-dependent ATP binding to β. Since, although it required the presence of $MgCl_2$ during the incubation, it remained bound to the β-subunit when centrifuged on Sephadex columns that were preequilibrated with buffer in the absence of $MgCl_2$ [8]. This could, however, be the reason for our failure to observe, in a similar set of experiments, a second, $MgCl_2$-dependent, binding site for ADP.

The experiments summarized in fig.1 were designed to test this possibility by including increasing concentrations of $MgCl_2$ in the incubation mixture as well as in the buffer used for preequilibration of the Sephadex columns before the elution-centrifugation step, that separates the β-subunit bound [^{3}H]ADP from the remaining free [^{3}H]ADP. The results indicate that this is indeed the case and under appropriate conditions two ADP binding sites can be revealed on the *R. rubrum* β-subunit. One of these sites does not require $MgCl_2$ and is not affected by it, since the same binding stoichiometry of 1 mol ADP/mol β was obtained when the $MgCl_2$ concentration during incubation was raised from 0 to 10 mM. ADP binding to the second site required the presence of $MgCl_2$ in the incubation mixture, being optimal when the $MgCl_2$ to ADP ratio was between 0.5 to 1.0, but it could be detected only when $MgCl_2$ was

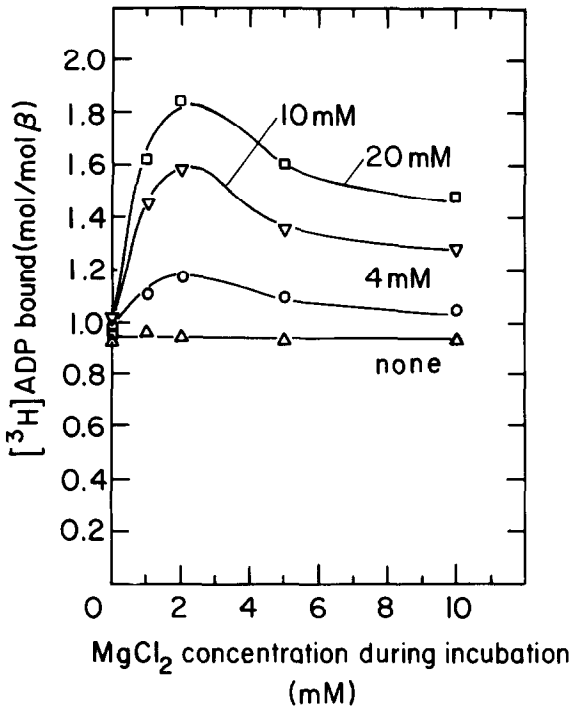

Fig.1. Dependence of ADP binding to a second site on the β-subunit on the presence of $MgCl_2$ in the incubation as well as the elution-centrifugation steps. β was incubated at 10 μM with 4 mM [^{3}H]ADP in TG buffer containing the indicated concentrations of $MgCl_2$, or 2 mM EDTA when no $MgCl_2$ was present. After 1 h aliquots from each incubation mixture were freed from unbound ADP by elution-centrifugation on a series of Sephadex G-50 columns equilibrated with TG buffer containing the following concentrations of $MgCl_2$: 0 mM (△), 4 mM (○), 10 mM (▽) and 20 mM (□). The binding stoichiometry was calculated as described in section 2.

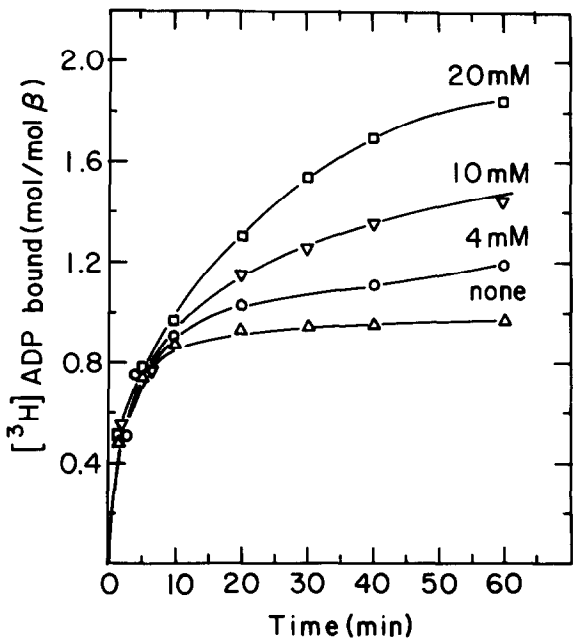

Fig.2. Time-dependence of ADP binding to the β-subunit as affected by the presence of $MgCl_2$ during the elution-centrifugation step. β was incubated at 10 μM with 4 mM [^{3}H]ADP in TG buffer containing 5 mM $MgCl_2$. At the time indicated, aliquots were freed from unbound ADP by elution-centrifugation on a series of Sephadex columns as described in fig.1.

included also in the preequilibration buffer used for the Sephadex columns (fig.1). These results indicate that the removal of $MgCl_2$ leads to a very rapid dissociation of ADP from this site. The concentration of $MgCl_2$ required for retaining all the ADP bound to this second site during the elution-centrifugation step is even higher than that required for the binding itself. Thus, although the presence of 2 mM $MgCl_2$ during incubation of β with 4 mM ADP was enough for complete occupation of both sites, the inclusion of at least 20 mM $MgCl_2$ in the preequilibration buffer was required to avoid the dissociation of ADP from the second site during the centrifugation step (fig.1).

The time course of ADP binding to both sites on the β-subunit is illustrated in fig.2. When $MgCl_2$ was present only in the incubation mixture, the binding of ADP reached a maximal level of $\approx$0.9 mol/mol β after about 10 min of incubation with a $t_{1/2}$ of 3 min. Addition of $MgCl_2$ also to the preequilibration buffer had no effect on the kinetics of ADP binding to this site, but revealed a second, much slower, phase of ADP binding, that was dependent on the concentration of $MgCl_2$ in the preequilibration buffer. When 20 mM $MgCl_2$ was added to the buffer, this second phase, which reached equilibrium binding only after 1 h with a $t_{1/2}$ of about 25 min, led to binding of an additional mol ADP/mol β.

The existence of two binding sites for ADP on the β-subunit was clearly demonstrated in experiments where β was incubated without and with 5 mM $MgCl_2$, in the presence of a wide range of ADP concentrations, and ADP binding was tested after elution-centrifugation on Sephadex columns

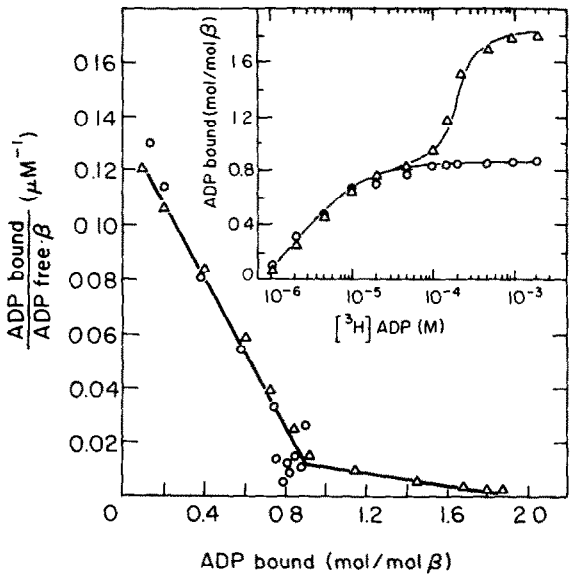

Fig.3. Scatchard plot analysis of ADP binding to the β-subunit. β was incubated at 10 μM with the indicated concentrations of [^{3}H]ADP in TG buffer containing either 5 mM $MgCl_2$ (△) or 2 mM EDTA in the absence of $MgCl_2$ (○). After 1 h aliquots from each incubation were freed from unbound ADP by elution-centrifugation on Sephadex columns equilibrated with TG buffer containing 20 mM $MgCl_2$, and the effluents were assayed for bound ADP as described in section 2.

preequilibrated with 20 mM $MgCl_2$ (fig.3, inset). When incubated in the absence of $MgCl_2$ about 1 mol of binding site/mol β was titrated, showing a monophasic binding curve that saturates at 50 μM ADP. The presence of $MgCl_2$ during both the incubation and centrifugation steps results in a biphasic binding curve. The first phase being identical to that observed in the absence of $MgCl_2$ during the incubation, whereas the second phase saturated only around 2 mM ADP, reaching a maximal level of about 1.8 mol ADP bound/mol β. A Scatchard plot analysis of the binding data (fig.3) reveals two binding sites, a high-affinity site with a K_d of 6.7 μM and a low-affinity site with a K_d of 80 μM. This K_d is identical to the K_m for ADP, measured during ATP synthesis with spinach thylakoids [17].

The results presented here regarding the kinetics and binding stoichiometry of ADP to the β-subunit are very similar to those earlier observed for ATP [8]. Both nucleotides bind, with similar K_d values, to two sites on β: to a high-affinity $MgCl_2$-independent site and to a low-affinity $MgCl_2$-dependent site. Furthermore, with both nucleotides the K_d values for the low-affinity site are very similar to the respective K_m values measured for ATP hydrolysis in *R. rubrum* chromatophores [18] and for ADP phosphorylation in spinach thylakoids [17].

One important difference has, however, emerged from the comparison of the binding properties of ATP and ADP to the β-subunit. ATP forms a very stable Mg–ATP complex at the low-affinity binding site in the β-subunit, whereas ADP forms an unstable complex that dissociates very rapidly. The remarkable stability of the Mg–ATP–β complex may be due to the presence of the γ phosphoryl group, which could react as an additional anchoring point for ATP at the binding site. We have recently obtained experimental evidence for this suggestion when studying the effect of ADP and ATP on the P_i binding site in the *R. rubrum* β-subunit [10].

Our observation, that the Mg–ADP–β complex is unstable, dissociating very rapidly upon removal of $MgCl_2$ after the binding step, might explain earlier reported data on the poor binding of nucleotides to the isolated *E. coli* β-subunit [5,9] and of P_i to CF_1 [19,20], if also in these cases an unstable Mg–ligand–protein complex is formed. Indeed, in all these reports $MgCl_2$, even when present during the incubation of the protein with the ligand, was removed when separating the bound and free ligands. It might, therefore, be worthwhile to repeat these experiments using high concentrations of $MgCl_2$ during the separation step.

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