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Inhibition of chloroplast CF_1-ATPase by vanadate

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Inhibition of ATPase activity by vanadate, having $K_{1/2}$ of 0.5 mM, was demonstrated in the CF_1-ATPase. The Ca^{2+}-dependent ATPase activity of the isolated enzyme was inhibited in an allosteric manner by vanadate with a Hill coefficient of 3.19 ± 0.6. Vanadate also inhibited ATPase and Pi–ATP exchange activities of the chloroplast membrane-bound enzyme. Using ^{51}V NMR it was demonstrated that ATP caused partial release of about 1.87 equivalents while ADP caused additional binding of approximately 1.46 equivalents of vanadate, when added to a solution containing CF_1 equilibrated with vanadate. The relevance of these results to a possible involvement of a pentacovalent phosphate as transition state intermediate in the hydrolysis of ATP by CF_1-ATPase is discussed.

Transition state; Enzyme mechanism; ATP synthase; Proton pump; ^{51}V NMR

1. INTRODUCTION

The H^+-ATPase in the chloroplast membrane utilizes an electrochemical potential of protons for the synthesis of ATP from ADP and Pi [1]. Its catalytic sector (CF_1) is a complex having a subunit stoichiometry of 3α, 3β, γ, δ, and ε [2]. The active site of the enzyme is believed to be located on the β subunits, as indicated by covalent binding of nucleotide analogues [3], by the high homology of β subunits from various organisms and by the conserved region in the vicinity of the nucleotide binding sequence [4]. Catalysis in the F-type ATPase was not shown to proceed through the formation of a covalently bound phosphate intermediate. However, other ion pumps, such as the P-type Ca^{2+}-ATPase, the Na^+/K^+-ATPase or the plasma membrane H^+-ATPase [5], form a covalently bound phosphate intermediate. In these enzymes a pentacovalent phosphorus was assumed to be a transient state intermediate since they were inhibited by the Pi analogue, vanadate [6]. Vanadate tends to form a pentacovalent intermediate during the formation of esters or anhydride bonds. The formation of a trigonal-bipyramidal coordination geometry about vanadate was shown by neutron diffraction of the crystalline uridine–vanadate–ribonuclease complex [7]. However, myosin ATPase, which does not form a covalently bound phosphate intermediate, was also shown to be inhibited by vanadate providing ADP was present [8].

Direct measurements by X-ray absorption of the metal at the three cooperative interacting sites revealed the existence of a ternary complex of enzyme, Mn^{2+} and ATP at these sites in CF_1 [9]. Based on the structure of the metal–ATP complex in the active sites and on the known chemistry of ATP hydrolysis in other types of ATPases, it might be expected that the catalysis in F-type ATPases also proceeded through a pentacovalent phosphate intermediate. Yet, to our knowledge [10,11], vanadate has not been shown to inhibit F-type ATPase. In this work we have shown that vanadate inhibited the activity of CF_1-ATPase, and its mode of binding to the enzyme was studied. The results were interpreted to suggest the involvement of a pentacovalent phosphorus as a transition state intermediate in reactions catalyzed by CF_1-ATPase.

Abbreviations: Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; DTT, dithiothreitol; CF_1, coupling factor 1 of chloroplast H^+-ATPase; Chl, chlorophyll; NMR, nuclear magnetic resonance.

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2. MATERIALS AND METHODS

2.1. *ATPase activity*

CF_1 was isolated from chloroplasts [12] prepared from lettuce (vr. romane) leaves as earlier described [13] and protein concentration was determined [14]. Ca^{2+}-dependent ATPase activity was assayed in a medium containing: 40 mM Tricine–NaOH, pH 8, 3 mM ATP, 3 mM $CaCl_2$ and 12 μg heat-activated [15] CF_1 in a total volume of 1 ml for 1 min at 37°C. Spectrophotometric determination of the released Pi was used [16]. Mg^{2+}-dependent activity was assayed in a medium containing: 40 mM Tricine–NaOH, pH 8, 10 mM ATP, 10 mM $MgCl_2$, 100 mM Na_2SO_3 and 12 μg CF_1, in a total volume of 1 ml for 5 min at 37°C [16]. ATPase and Pi–ATP exchange activities were assayed in light-triggered chloroplasts as earlier described [13]. [^{32}P]ATP was determined by scintillation counting following the removal of Pi as ammonium molybdate complex by precipitation [17].

2.2. *NMR measurements of ^{51}V*

These were performed in a Brukner W M 360 spectrometer, equipped with a multinuclear probe at 94.63 MHz. Pulse widths of 50°, sweep widths of 20 kHz and acquisition times of 0.13 s, with a recycle delay of 0.05 s were used. A line-broadening of 20 or 50 Hz were

applied to all spectra before Fourier transforming to the frequency domain with the use of a 4K data set zero-field to 8K. The sample (1 or 2 ml) contained 40 mM Tris-HCl, pH 8, and 0.165 mM CF_1-protein.

3. RESULTS

3.1. *Inhibition of ATPase activity by vanadate in CF_1*

The failure to demonstrate inhibition of F-type ATPases by vanadate in mitochondria [10,11] could have been a result of uninducive experimental conditions. Indeed, it was found that the Ca^{2+}-dependent ATPase activity of the heat-activated CF_1 could be inhibited by vanadate at a concentration range of 0.1–2.0 mM (Fig. 1). At 3 mM ATP there was an increase in ATPase activity as a function of the increase in $CaCl_2$ concentration (Fig. 2). The extent of vanadate inhibition sharply increased above 1.5 mM $CaCl_2$. Using a K_d value of 0.1 mM for the Ca–ATP complex the concentration of free Ca^{2+} ions was calculated. It was found that the onset of the inhibition coincided with a concentration larger than 0.1 mM of the free metal ion. Ca^{2+}-dependent ATPase activity gave a saturation curve having a slight sigmoidal shape in response to increasing concentrations of ATP, with 3 mM $CaCl_2$ (Fig. 2). However, a decrease in the extent of inhibition by vanadate occurred with increasing ATP concentrations. As a result, a clear sigmoidal response curve was obtained in the presence of vanadate (Fig. 3). Hill coefficient was calculated using Ln S vs. Ln $[v/(V_{max}-v)]$ (Fig. 3, insert), where the substrate (S)ATP, the rate of activity (v) and the maximal rate of activity (V_{max}) were plotted. The Hill coefficient progressively increased from 1.28 ± 0.27 to 3.19 ± 0.6 when vanadate concentration was increased from 0.6 to 1.2 mM. The simplest interpretation of such sigmoidity would assume an allosteric inhibition of the activity with respect to ATP. The membrane-bound CF_1 catalyzes light-driven phosphorylation of ADP to ATP as well as light-triggered ATPase and Pi–ATP exchange activities. It was found that both light-triggered activities were inhibited by vanadate to about the same extent as ATPase in the isolated CF_1 (Table I). However, photophosphorylation was insensitive to vanadate under the experimental conditions used.

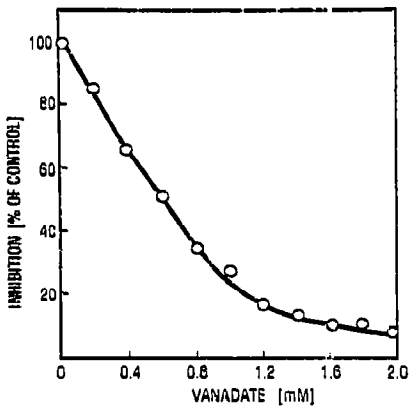

Fig. 1. Inhibition of ATPase activity by vanadate. Ca^{2+}-dependent ATPase activity of the heat-activated CF_1 was assayed with 0.25 mM ATP, 3 mM $CaCl_2$ and varying concentrations of vanadate, as indicated. The rate of activity in the absence of added vanadate was 2.9 ± 0.2 μmol/mg CF_1/min.

3.2. *Studies of ^{51}V NMR of CF_1-bound vanadate*

The assay conditions and the concentrations of the reagents used in the NMR measurements were similar to those used in the assay of enzyme activity except for an increase in the concentration of CF_1, designed to amplify possible bound species of vanadate. A major resonance at −537 ppm, corresponding to monovanadate [18], was observed in solution (Fig. 4A). Two low intensity resonances at −550 ppm and at −565 ppm, which corresponded to the divanadate and the tetravanadate, respectively [18], were only 1% of the total vanadate. A major change, however, occurred on addition of CF_1 to a solution of vanadate. The signal of the free vanadate was drastically reduced while a new broad resonance at −498 ppm appeared (Fig. 4C). This signal was assigned to the enzyme-bound vanadate.

A possible interaction between the enzyme-bound va-

Table I

The effect of vanadate on the activity of the isolated and the membrane-bound CF_1

	CF_1 (μmol/mg/min)		Thylakoids (μmol/mg Chl/h)		
	ATPase		ATPase	Pi–ATP exchange	Synthesis
	Ca^{2+}	Mg^{2+}	Mg^{2+}	Mg^{2+}	Mg^{2+}
None	12.3	17.5	60.7	18.3	343.5
Vanadate (1 mM)	3.1	8.2	10.9	10.1	351.7
Inhibition (%)	74.8	53.1	82.0	44.8	None

The inhibition by vanadate was measured in heat-activated isolated CF_1 or in light-triggered enzyme bound to the chloroplast membrane. The rates of activity were calculated on the basis of protein and chlorophyll for CF_1 and the chloroplasts, respectively.

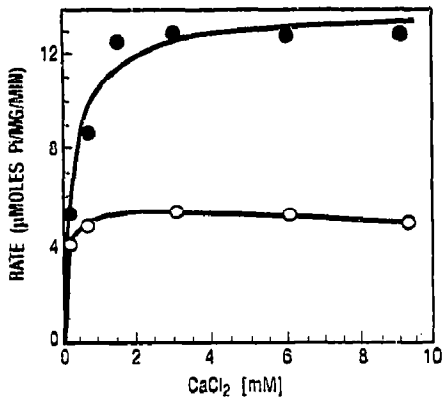

Fig. 2. The effect of $CaCl_2$ on the inhibition of ATPase activity by vanadate. ATPase activity was assayed in heat-activated CF_1 in the presence of 3 mM ATP and without (●) or with (○) 1.2 mM vanadate.

nadate, Ca^2 and ATP was investigated. On addition of $CaCl_2$ and ATP only a very slight decrease in all three signals of free vanadate in solution were observed (Fig. 4B). Only a slight decrease was also observed in the signal of the enzyme-bound vanadate on addition of $CaCl_2$ (Fig. 4D and E). However, 0.5 mM ATP caused an almost 4-fold increase in the signal of the free vanadate with just a minor change in the signal of that bound to the enzyme (Fig. 4F). With CF_1 concentration at 0.165 mM, three binding sites on the enzyme having a K_d in the 1 μM range [1] were almost saturated at this concentration of ATP. Thus it seemed that binding of ATP caused a release of some of the bound vanadate from the enzyme. The amount of the released vanadate was calculated from the integration of the area of the signal to be equivalent to approximately 1.87 binding sites. Contrary to the effect of ATP, addition of ADP, in the presence of Ca^{2+} ions, increased the signal of the bound vanadate while causing a small decrease in the

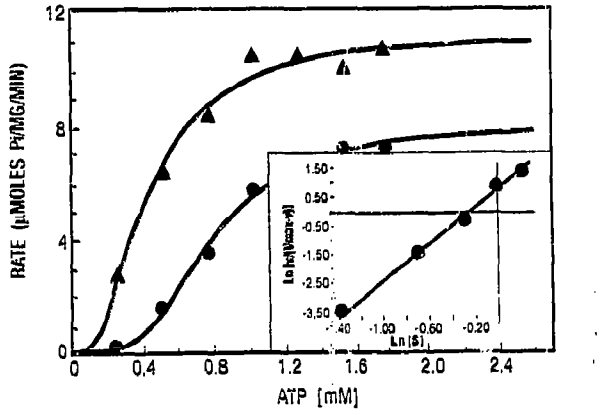

Fig. 3. Allosteric effect. ATPase activity was assayed in heat-activated CF_1 in the presence of 3 mM $CaCl_2$ and varying concentrations of ATP without (▲) and with (●) 1.2 mM vanadate. The insert is a Hill plot of the activity in the presence of vanadate. Hill coefficient was 3.19 ± 0.6 and the V_{max} values of 11.4 ± 0.6 and 8.2 ± 0.5 (μmol/mg CF_1/min) without and with vanadate, respectively, were calculated.

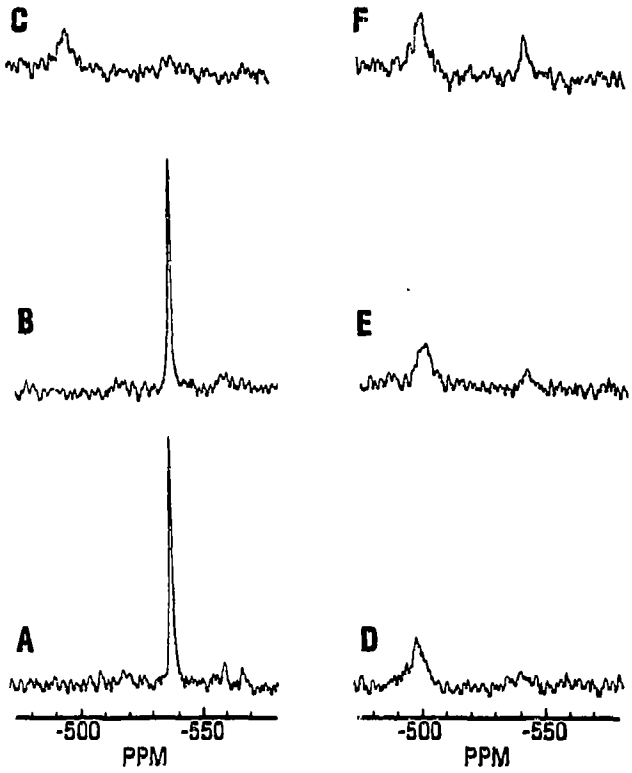

Fig. 4. ^{51}V NMR spectra of vanadate in solution with and without CF_1. The samples contained vanadate at a concentration of 1 mM (left panel) and 1.5 mM (right panel). Other additions were: A, none; B, 3 mM ATP and 3 mM $CaCl_2$; C–F, 0.165 mM CF_1; E, 3 mM $CaCl_2$; F, 3 mM $CaCl_2$ and 0.5 mM ATP. The resonances at −498, −537, −558 and −565 ppm were assigned to bound CF_1 and to free monovanadate, divanadate and tetravanadate, respectively.

signal of the free vanadate (not shown), indicating an increase in the binding of vanadate on binding of ADP to the enzyme. ADP caused additional binding which was equivalent to approximately 1.46 sites on the enzyme.

4. DISCUSSION

4.1. *Allosteric effect*

The similarity in the K_m for phosphate in photophosphorylation and in the $K_{1/2}$ for inhibition by the phosphate analogue vanadate might indicate that they both bind at the same site to CF_1. Yet phosphate did not inhibit ATPase activity [13] while vanadate did. It is possible that the inhibition was caused by a formation of an pentacovalent vanadyl ADP adduct which abstracted the binding of ATP to the catalytic sites. A formation of such an adduct was suggested to inhibit myosin ATPase [8]. Evidence which supports binding of the inhibitor to the active site came from the sigmoidal response curve observed when the rate of Ca^{2+}-dependent ATPase activity was measured as a function of increasing concentrations of ATP, in the presence of vanadate. A strong interaction among the vanadate bind-

ing sites was indicated by the observed Hill coefficient of 3.19 ± 0.5. In an interpretation of such an effect it could be assumed that vanadate was bound to three active sites on the enzyme, one at each of the β subunits of the enzyme. Subunit interaction which was earlier shown to occur among these subunits [1,4] could convey a cooperative interaction which caused the sigmoidal type of response in the inhibition by vanadate. The decrease in the concentration of the free metal at increasing concentrations of ATP did not cause the sigmoidal response. A decrease in the inhibition by vanadate was expected only when the free metal decreased below 0.1 mM. However, the sigmoidal response occurred above a concentration of 1.4 mM of the free Ca^{2+} ions (Fig. 3).

Exclusion of vanadate interaction with sites other than the active ones came from studies of the Mg^{2+}-dependent activity. It seemed that vanadate probably did not bind to the same anionic binding site as the sulfite. The absence of interaction was indicated by the observation that vanadate was as effective an inhibitor in the presence as in the absence of sulfite and no change in the extent of the inhibition could be observed when ATPase activity was assayed with varying concentrations of sulfite (not shown). Vanadate did not inhibit the activity by indirectly changing the state of activation either in the soluble or in the membrane-bound enzyme. In the isolated enzyme removal of vanadate by dilution restored the activity, indicating that the enzyme remained active. In the membrane-bound CF_1 vanadate was shown not to effect the rate of decay of the light-triggered state required for ATP hydrolysis.

4.2. ^{51}V NMR

^{51}V NMR studies enabled the observation of a release of approximately two equivalents of the bound vanadate on addition of ATP and the binding of a similar amount of free vanadate on addition of ADP. Although the quantitative estimates were based on the integration of the area under the signals which had 25% background noise, the approximations could give an idea of the relative magnitudes and the direction of the changes. It is tempting to speculate that ADP enhanced the binding of vanadate by formation of an ADP vanadyl pentacovalent intermediate at the active site and that the γ-phosphate of ATP caused a release of the vanadate which was bound at the phosphate binding site. The preliminary data obtained from direct measurements of the ternary complex of CF_1, ADP, Mn^{2+} and vanadate by X-ray absorption were in harmony with this suggestion. In these unpublished observations vanadate was shown to bind within 3.5 Å to the ADP phosphorus at the Mn·nucleotide sites in the enzyme and assumed higher symmetry, characteristic of a pentacovalent intermediate rather than to the tetrahedral configuration of vanadate in solution.

It can be speculated that the ADP produced during ATP hydrolysis interacted to form a pentacovalent vanadyl adduct at the active site. Such an adduct would have a higher affinity than ATP and interfere with reversible reactions such as ATP hydrolysis and Pi–ATP exchange. However, it did not affect ATP synthesis just as ATP did not inhibit this reaction.

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