Vanadate, a Transition State Inhibitor of Chloroplast CF$_1$-ATPase*

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The activity of CF$_1$-ATPase was inhibited by vanadate in an allosteric manner with respect to CaATP as substrate. The cooperative interaction was enhanced by preincubation of the enzyme in the presence of ADP and Ca$^{2+}$ ions and of free divalent metal ions during assay of the activity. The strongest cooperative interaction with a Hill coefficient of 5.3 ± 0.1 was found when the reaction was stopped after 30 s, before steady state was reached. Under these conditions, the concentration of an exchangeable ADP, tightly bound to one of the active sites on the enzyme, was shown to be the highest. A $K_H$ of 12.4 ± 1.2 $\mu$M for vanadate inhibition was determined under these conditions. Direct measurements with the aid of $^3$V NMR indicated that vanadate binds to CF$_1$ in the presence of Ca$^{2+}$ and ADP in a positive cooperative manner with a Hill coefficient of 2.3 ± 0.2 and an average $K_H$ of 0.3 ± 0.04 nM. It was suggested that a formation of a pentacovalent vanadyl-ADP at the active site caused the inhibition. Vanadyl-ADP was suggested to be a strong inhibitor, being an analogue of a pentacovalent phosphoryl-ADP, which is proposed to be the transition state intermediate of CF$_1$.

The proton-translocating reversible ATPase contains two major sections: hydrophobic proteins (CF$_0$), situated in the membrane and serving as a proton channel, to which coupling factor 1 (CF$_1$) is attached (1). The catalytic sector (CF$_1$) of the chloroplast H$^+$/ATPase is composed of five types of subunits designated: $\alpha$, $\beta$, $\gamma$, $\delta$, and $\epsilon$ (2). Several lines of evidence indicating the $\beta$ subunits as the possible location of the active site included a high sequence homology of the $\beta$ subunits from various organisms, a conserved region in the vicinity of the nucleotide binding sequence (3), and affinity labeling studies with ADP and ATP analogues (4). CF$_1$ was suggested to have a total of six nucleotide binding sites (4, 5). Three of the sites were assigned to the $\beta$ subunits and three to the $\alpha$ at the interface of the $\beta$ subunits. Based on specificity, capacity to synthesize (6) or to hydrolyze (7) ATP, and affinity labeling studies (8), the catalytic sites were suggested to be on the $\beta$ subunits while noncatalytic sites were assigned to the $\alpha$ subunits. The mechanism of the ATP synthase is probably described best by the "alternating site" hypothesis (9). ATP is formed from tightly bound ADP and P$_i$ or hydrolyzed from bound ATP, alternately, at the three catalytic sites on the F$_1$. The release of the product is induced by a conformational change caused either by the electrochemical potential of protons across the membrane during synthesis or by cooperative interaction caused by binding of ATP to other sites during hydrolysis. The sequence of reactions is coordinated through cooperative interaction among the three active sites of the enzyme. Interactions among the subunits were exemplified by the ability of ADP, bound to a single site, to quench the activated state of CF$_1$ (10) and to modulate ATPase activity in the isolated CF$_1$ (11). $H_2^{18}$O-ATP exchange was also modulated by cooperative binding of ATP (9). Support for this hypothesis came from the finding of ATP hydrolysis with an equilibrium constant of approximately 1 (12) and a concomitant catalysis of $H_2^{18}$O-ATP exchange which occurred when the nucleotides were tightly bound to a single site on the enzyme.

Catalysis in the F-type ATPase was shown to proceed through direct in-line transfer of phosphoric residue between ADP and water and not through the formation of a covalently bound phosphate intermediate (13). However, other ion pumps such as the P-type Ca$^{2+}$-ATPase, the Na$^+$/K$^+$-ATPase, or the plasma membrane H$^+$-ATPase (14) form a covalently bound phosphate intermediate. In these enzymes, a pentacovalent phosphorus was assumed to be a transition state intermediate since they are readily inhibited by the P$_i$ analogue vanadate (15). Myosin and dynein ATPases, as well as ribonuclease, phosphoglucomutase, alkaline phosphatase, aryl sulfatase, and phosphoglucomutase belong to another group of enzymes which are inhibited by a vanadyl adduct of substrate or product (16). The formation of a trigonal-bipyramidal coordination geometry about vanadate was shown by neutron diffraction of the crystalline uridine-vanadate-ribonuclease complex (17).

Direct measurements by X-ray absorption of the metal at the three cooperative interacting sites revealed the existence of the ternary complex of enzyme, Mn$^{2+}$, and ATP at these sites in CF$_1$ (18, 19). 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. The configuration of the oxygens about the phosphate indicates an inversion at the $\gamma$-phosphate of ATP during hydrolysis. Such an inversion could indicate that the hydrolysis proceeded through a formation of pentacovalent intermediate. Vanadate is a weak competitive inhibitor of oxidative phosphorylation (20), but it failed to inhibit the activity of mitochondrial F-ATPase (21). In preliminary work we have shown (22) that vanadate inhibited the activity of CF$_1$-ATPase with half-maximal effect at 0.5 mM. In this work it was shown that in the presence of CaADP micromolar concentrations of vanadate strongly inhibited the activity. The results were interpreted to suggest the involvement of a pentacovalent phosphorus as a transition state intermediate in reactions catalyzed by CF$_1$-ATPase.

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$^1$The abbreviations used are: CF$_0$, coupling factor 1 of chloroplast H$^+$/ATPase; Tricine, N-tris(hydroxyethyl)methylglycine; MOPS, 3-(N-morpholino)propanesulfonic acid.
ATPase Activity—CF₁ was isolated from chloroplasts prepared from lettuce (var. romaine) leaves as earlier described (23). Protein concentration was determined as in Ref. 24. Stored CF₁ was passed through a centrifuged Sephadex G-50 column as above. ATPase activity was assayed in a medium containing 50 mM sodium Tricine, pH 8, and 1 mM EDTA. Latent CF₁ was heat-activated at 64 °C in the presence of 5 mM dithiothreitol and 40 mM ATP as described (25). Following activation, the enzyme was passed through a centrifuged Sephadex G-50 column as above. ATPase activity was assayed in a medium containing 50 mM sodium Tricine, pH 8, 2-25 μg/ml heat-activated CF₁, and CaATP, and free Ca²⁺ as indicated. The reaction time was varied from 30 s to 10 min, as indicated. Dissociation constants of 0.17 mM for CaATP and CaADP (26), respectively, were used for calculations of the concentrations of the complexes and of free ATP, ADP, and Ca²⁺ ions. The released Pi was determined by the spectrophotometric method (27).

Vanadate Composition—Aqueous solutions of vanadate anions contain several oligomers including monomers, dimers, and tetramers (28). ⁶¹V NMR measurements were used to determine the concentration of various oligomers in 50 mM Tris-Mops, pH 6, at pH 9.5, at 0.1 to 2 mM vanadate (Figs. 4 and 6). Association constants of 0.07 mm⁻¹ and 0.037 mm⁻³ were calculated for the formation of dimeric and tetrameric vanadate from monomeric vanadate at pH 8 as in Ref. 28. The composition of vanadate in the solutions calculated by these constants was in agreement with the experimental data obtained from ⁶¹V NMR measurements (Fig. 4, inset).

Data Analysis—The kinetic parameters of ATPase activity of CF₁ were determined using the Dixon (Eq. 1), Hill (Eq. 2), and Michaelis-Menten (Eq. 3) equations. Binding data were plotted according to the Scatchard (Eq. 4) equation. The binding constants for the interacting binding sites were analyzed by a semiempirical method as previously described (29). Data were fit to the equations by the “KaleidaGraph 2.1.1,” a graphic software system by Synergy Software, Reading, PA. Theoretical binding and ATPase activity plots were also drawn by the “KaleidaGraph” graphic software.

\[
\begin{align*}
\frac{1}{v} &= \frac{1}{V_{\text{max}}} + \frac{[I]}{K_i} \quad (\text{Eq. 1}) \\
\frac{v}{V_{\text{max}}} &= \frac{[S]}{K_m + [S]} \quad (\text{Eq. 2}) \\
\frac{v}{V_{\text{max}}} &= \frac{[S]}{K_m + [S]} \quad (\text{Eq. 3})
\end{align*}
\]

Where \( v \) and \( V_{\text{max}} \) are specific activities of CF₁, \( [S] \) and \( [I] \) are substrate and inhibitor concentrations, \( K_m \) is the Michaelis constant, and \( n \) is the Hill coefficient.

\[
\nu = \frac{N_k C}{1 + K_c C} \quad (\text{Eq. 4})
\]

Where \( \nu \) is moles of vanadate bound per mol of CF₁, \( N_k \) is the number of interacting binding sites, \( C \) is the concentration of free vanadate, and \( n \) is the Hill coefficient.

Effect of Free Ca²⁺ Ion on Inhibition by Vanadate—In preliminary work we have shown (22) that vanadate, an analogue of phosphate, showed an allosteric inhibition of ATPase activity of CF₁. Although half-maximal inhibition was obtained at 0.5 mM vanadate, direct binding measurements by ⁶¹V NMR spectroscopy indicated that in the presence of ADP and a divalent cation, vanadate binds to the enzyme at a much lower dissociation constant. It was also shown that the inhibition was enhanced in the presence of a free divalent cation at concentrations higher than 0.1 mM. Initially, the effect of free divalent cation on the inhibition by vanadate was explored. The activity was assayed in the presence of Ca²⁺ ions which are analogues of the physiological Mg²⁺ ions, because free Ca²⁺ ions are much less inhibitory than free Mg²⁺ (1). The less inhibitory ions were preferred in this study in order to minimize interference with the inhibition by vanadate. Yet ATPase activity was shown to be equally sensitive to vanadate in the presence of each of these cations (22). The activity was measured at various concentrations of free Ca²⁺ ions and constant concentration of CaATP in the presence and in the absence of vanadate. The results were presented as the percent of activity left in the presence of vanadate as a function of the concentration of free Ca²⁺ ions (Fig. 1). Vanadate inhibited the activity even at very low concentrations of free divalent cation. However, the inhibition was strongly enhanced by increasing the concentrations of the free cation. A dissociation constant of 1.7 ± 0.2 μM was calculated for free Ca²⁺ ions as enhancers of the inhibition caused by the vanadate ions using the Dixon plot (Fig. 1, inset). This value was similar to the dissociation constant of binding of divalent cations to three interacting sites on CF₁ (30). It is possible that vanadate inhibition was enhanced as a result of binding of cations to these sites on the enzyme.

Allosteric Inhibition by ADP and Vanadate—The effect of ADP on vanadate inhibition was therefore examined following
preincubation of the enzyme in the presence of Ca\(^{2+}\) and with 0.5 mM free Ca\(^{2+}\) ions during the assay of the activity. In the absence of ADP, or vanadate, the activity as a function of CaATP concentration gave a hyperbolic line (Fig. 2). However, both ADP and vanadate inhibited the activity in a sigmoidal manner with Hill coefficients of 1.6 ± 0.2 and 2.1 ± 0.1, respectively (Table I). Much stronger inhibition was obtained in the presence of both vanadate and ADP. The inhibition was allosteric with CaATP as substrate with a Hill coefficient of 5.3 ± 0.1 (Table I), indicating strong subunit interaction. Although the effects of the two inhibitors on \(V_{\text{max}}\) were additive, a strong synergistic effect was seen at low substrate concentrations.

These results were interpreted to suggest that the enzyme sensitivity to inhibition by vanadate greatly increased by binding of divalent cations and ADP. Divalent ions and ADP were shown to increase the pre-steady state lag of ATPase activity (31). The possibility that the sigmoidal response was a result of this lag was therefore evaluated. The rate of pre-steady state activity was low and was accelerated on addition of CaATP at a rate constant of 0.05 s\(^{-1}\) until steady state rate was reached when CF\(_1\) was preincubated with divalent cations (31). The rate constant for the acceleration was also shown to increase as a function of CaATP concentration. The inhibition by ADP and divalent cations was therefore expected to be stronger at low substrate concentrations resulting in a sigmoidal dependence on substrate concentration. A more pronounced sigmoidity was also expected when the reaction was stopped before steady state was reached. The Hill coefficient values of ATPase activity measured in the presence of ADP, vanadate, or vanadate and ADP were 1.3 ± 0.1, 1.0, and 1.5 ± 0.1, respectively, when the reaction time was 10 min (Table I). However, the Hill coefficients measured for 30 s of reaction were 1.6 ± 0.2, 2.1 ± 0.1, and 5.3 ± 0.1 for similar effector combinations, respectively. A pronounced increase in cooperative interaction was observed at pre-steady state activity.

The pre-steady state acceleration of ATPase activity was also shown to be slower following preincubation of the enzyme with divalent cations (31). Assuming that the sigmoidal response was due to the pre-steady lag in the activity, preincubation of the enzyme in the presence of divalent cations should also increase the sigmoidal effect of ADP and vanadate. Indeed, in the presence of ADP or vanadate or ADP and vanadate, Hill coefficient values of 1.7 ± 0.2, 1.30 ± 0.3, and 2.5 ± 0.6 were obtained, respectively, when the enzyme was preincubated in the presence of Ca\(^{2+}\) ions and the reaction started with the addition of ATP, compared to Hill coefficient values of 1.1 ± 0.2, 1, and 1.8 ± 0.2, respectively when the reaction was started with the addition of CF\(_1\) without preincubation (Table II).

**Enhancement of Vanadate Inhibition by CaADP**—The involvement of Ca\(^{2+}\) ions and ADP in the enhancement of the inhibition by vanadate could be a result of binding of a CaADP complex to the enzyme. This latter possibility was examined by determination of the \(K_i\) for inhibition of ATPase activity by CaADP at various concentrations of vanadate. At each

![Inhibition of ATPase activity by ADP and vanadate](image)

**TABLE I**

The effect of the length of the reaction time of ATPase activity on the Hill coefficient

| Reaction time | Hill coefficient | \(V_{\text{max}}\) | Hill coefficient | \(V_{\text{max}}\) |
|---------------|------------------|------------------|------------------|------------------|
| 10 min        | ADP              | 1.3 ± 0.1        | 13.5 ± 1.0       | 4.3 ± 0.3        |
|               | Vanadate         | 1.0 ± 0.0        | 7.0 ± 0.7        | 2.1 ± 0.1        |
|               | ADP and vanadate | 1.5 ± 0.1        | 1.9 ± 0.2        | 5.3 ± 0.1        |
| 0.5 min       | None             | 1.0 ± 0.0        | 12.0 ± 0.3       | 10.4 ± 0.8       |

**TABLE II**

The effect of the preincubation of CF\(_1\), with free Ca\(^{2+}\) ions on the Hill coefficient

Data were analyzed using the Michaelis-Menten or Hill equations as described under “Experimental Procedures.” The Hill coefficient for data fit to the Michaelis-Menten equation is equal to 1. CaATP concentration was varied from 0.03 mM to 4 mM with 1 mM free Ca\(^{2+}\) ions. Both ADP and vanadate concentrations were 0.5 mM. Heat-activated CF\(_1\) was preincubated at 37°C for 5 min. The reaction was started by addition of ATP. \(V_{\text{max}}\) in micromoles × mg\(^{-1}\) × min\(^{-1}\). ATPase activity was measured for 30 s as described under “Experimental Procedures.”

| Preincubulation time | Hill coefficient | \(V_{\text{max}}\) | Hill coefficient | \(V_{\text{max}}\) |
|----------------------|------------------|------------------|------------------|------------------|
| 5 min                | None             | 1.0 ± 0.0        | 12.6 ± 0.3       | 10.2 ± 0.8       |
|                      | ADP              | 1.1 ± 0.2        | 11.4 ± 1.6       | 3.5 ± 2.0        |
|                      | Vanadate         | 1.0 ± 0.0        | 9.0 ± 0.3        | 6.1 ± 2.1        |
|                      | ADP and vanadate | 1.8 ± 0.2        | 8.2 ± 0.7        | 2.5 ± 0.6        |
vanadate concentration, the activity was measured in the presence of varying CaADP concentrations and in the presence of constant concentration of CaATP and free Ca\(^{2+}\) ions. The Dixon plot used for determination of the \(K_i\) gave a straight line (data not shown) indicating that CaADP is directly involved in the inhibition of the activity. Even very low concentrations of vanadate greatly enhanced the inhibition of the activity by CaADP (Fig. 3). However, since the solution of vanadate contains monovanadate and its polymers, it was important to verify the active species in the mixture. The concentrations of the various polymers of vanadate were determined by \(^{51}\)V NMR of the solutions used in the measurement of ATPase activity. Up to 1 mM total vanadate, there was an almost linear increase in monovanadate which is the major species in the solution. Significant concentrations of di- and tetravanadate were detected only above 0.5 mM total vanadate (Fig. 3, inset). Linear relations were obtained in a Dixon plot of the change in the \(K_i\) for CaADP as a function of monovanadate concentration (Fig. 4). The linear relation in the Dixon plot might indicate that monovanadate was the effective species which inhibited the activity in the presence of CaADP. A \(K_i\) of 12.4 ± 1.2 \(\mu\)M for vanadate inhibition was calculated from the Dixon equation. This value was approximately 30-fold lower than the initial \(K_i\) for inhibition obtained in the absence of CaADP (22). This should be compared to a nonlinear Dixon plot obtained when the same effect was determined as a function of either divanadate or tetravanadate (Fig. 4). The nonlinearity indicates no direct relation between the change in the concentrations of the dimer and the tetramer and the inhibition of the activity.

A similar approach was used for determination of the active inhibitor in the mixture of Ca\(^{2+}\) and ADP. The \(K_i\) for vanadate inhibition was determined at increasing CaADP concentrations. There was a decrease in the \(K_i\) for vanadate with an increase in the concentration of CaADP. The Dixon plot of the change in the \(K_i\) for vanadate as a function of CaADP concentration was linear (data not shown). From the Dixon plot, a dissociation constant of 16.9 ± 1.2 \(\mu\)M for the effect of CaADP was determined. From the same experimental results, \(K_i\) values of 48.2 ± 6.0 \(\mu\)M and 28.8 ± 3.0 \(\mu\)M for total and free ADP, respectively, were calculated. The fact that the \(K_i\) values for either total or free ADP were higher than those calculated for CaADP indicates that the latter was the active component in the mixture. These values should be compared to the much lower dissociation constant of 1.7 ± 0.2 \(\mu\)M obtained for the effect of free Ca\(^{2+}\) ions on the inhibition by vanadate.

The Effect of pH on the Inhibition of ATPase Activity by Vanadate—The relative concentrations of vanadate and its various polymers depend, among other factors, on the concentration of total vanadate, on the ionic strength, and on the pH of the solution. As the pH was increased from 6 to 9.5, the relative concentration of di- and tetravanadate decreased from about 30% to less than 1% while monovanadate increased accordingly (Fig. 5, inset). The inhibition of ATPase activity by vanadate increased with the elevation of the pH in correlation with the increase in the relative concentration of the monovanadate (Fig. 5). An increase in inhibition with the increase of the one of the species of vanadate supports the suggestion that monovanadate rather than the polyvanadates was involved in the inhibition of ATPase activity.

Binding of Vanadate to CF1—The \(K_i\) values of the inhibition of activity gave indirect measurement for the dissociation constant of binding of vanadate to the enzyme. The possibility of multiple interacting binding sites for vanadate was indicated from the sigmoidal activity response to ATP in the presence of ADP and Ca\(^{2+}\) ions. It was therefore of interest to evaluate the data by direct binding assay. Direct measurements of binding were performed by titration of activated CF\(_1\), in the presence of Ca\(^{2+}\) and ADP, with vanadate. At each concentration of added total vanadate, the concentrations of the free mono-, di-, and tetravanadate were determined by

![Figure 3](image-url)  
**Fig. 3.** Modulation by vanadate of the inhibition of ATPase activity by CaADP. The \(K_i\) of CaADP, as an inhibitor of ATPase activity, was plotted as a function of vanadate concentrations varying from 0 to 1 mM. At each vanadate concentration, the activity was measured in the presence of CaADP concentrations varying from 0 to 0.4 mM with 0.1 mM CaATP and 1 mM free Ca\(^{2+}\) ions. Following preincubation for 5 min, the reaction was started by the addition of ATP. ATPase activity was measured for 30 s as described under "Experimental Procedures." The \(K_i\) of CaADP at each of the vanadate concentrations was calculated by the Dixon equation. The composition of 0 to 2 mM vanadate in the solutions was determined by \(^{51}\)V NMR measurements as described under "Experimental Procedures." The calculated association constants for the formation of dimeric (\(D\)) and tetrameric (\(\Delta\)) vanadate from monomeric (\(\ominus\)) vanadate were used to fit the data (inset).

![Figure 4](image-url)  
**Fig. 4.** Inhibition of ATPase activity by monomeric, dimeric, and tetrameric vanadate. Reciprocal values of \(K_i\) of CaADP as a function of monomeric (\(\ominus\)), dimeric (\(D\)), and tetrameric (\(\Delta\)) vanadate concentrations are plotted. Experimental conditions are as in Fig. 3. The composition of vanadate solutions from 0.003 mM to 0.2 mM was determined and calculated by NMR measurements as described in Fig. 3 (inset).
Vanadate Inhibition of CF$_1$-ATPase

The Scatchard plot gave an isotherm which curved upward to a maximum and then descended nonlinearly (Fig. 6, inset). Using a semiempirical analysis (29), the contribution of noninteracting sites was subtracted from the total yielding a plot of the interacting site (Fig. 6). The data were fitted to three interacting sites with a Hill coefficient of 2.23 ± 0.2 and an average $K_d$ of 0.3 ± 0.03 mM. Additionally, approximately 30 weakly noninteracting binding sites having an approximate $K_d$ of 6 mM were calculated. There was a 5-fold increase in the $K_d$ of binding of vanadate to activated CF$_1$ without the addition of Ca$^{2+}$ and ADP. The fact that the $K_d$ for binding was lower than the $K_d$ of inhibition supports the suggestion that the $K_d$ was a result of the steady state concentration of the enzyme in transition state during the hydrolysis of ATP.

**Discussion**

Transition State Conformation—Based on preliminary evidence, we have suggested (22) that an inhibition of CF$_1$ by vanadate was caused by a formation of a pentacovalent vanadyl ADP adduct at the active site. Such a complex was suggested to be structurally similar to the transition state of ATP during hydrolysis and synthesis. A transition state analogue would be expected to bind to the enzyme more strongly than the substrate. Yet, vanadate was a weak inhibitor, having $K_d$ similar to the $K_d$ for phosphate (22). The rather high $K_d$ could be understood as vanadate was shown to behave as a reversible rather than a dead end inhibitor. In such a case, it was reasonable to assume that the $K_d$ might depend on the concentration of the enzyme which was in transition state conformation. In this state, nucleotides were shown to bind tightly to the active site of the enzyme (12). Some experimental evidence indicated that each of the three active sites alternately assumed this conformation during the catalytic cycle. Yet, before ATP was added to the activated CF$_1$, one tightly bound exchangeable molecule of ADP was found on the enzyme. It was possible that this ADP was bound to the subunit which assumed a transition state conformation and therefore was tightly bound. Nucleotide binding site 1 (7), where the exchangeable ADP was bound, was shown to be located on the β subunit (8) and to catalyze hydrolysis of bound ATP (7) and synthesis of ATP from bound ADP (6, 32). Therefore, this was suggested to be an active site of the enzyme. If this were the case, vanadate should bind most strongly to the exchangeable bound ADP. The inhibition of the ATPase activity by vanadate was therefore measured under experimental conditions known to increase the concentration of the bound ADP by slowing down its dissociation from the enzyme. Positive cooperative binding of divalent cations to three sites on the enzyme (30) slowed down the release of the exchangeable ADP caused by the onset of ATPase activity on addition of substrate (33, 34). The release of ADP occurred concomitantly and at the same rate constant as the pre-steady state acceleration of ATPase activity (30). It was assumed that following preincubation of the enzyme with divalent cations and if the reaction was ended before steady state was reached, the concentration of the enzyme-bound ADP would be high and that the inhibition by vanadate would be the strongest. Since the rate of acceleration of the activity depended also on the concentration of the substrate it was expected that a sigmoidal activity curve would be obtained as a function of ATP concentration. Indeed, in this work, a sigmoidal response with the Hill coefficient of 5.3 ± 0.1 in the presence of vanadate and ADP was observed. The high value for Hill coefficient indicated strong cooperative interaction among vanadate binding sites on the enzyme.

Correlation between Sigmoidity and the Concentration of the...
Bound Exchangeable ADP—The suggestion that the sigmoidal response was a consequence of conditions which favored transition state conformation was supported by a systematic evaluation of some of the factors expected to increase the concentration of the bound exchangeable ADP. Information was available from the numerous reports which dealt with this phenomenon in great detail (33-35). The major factors would be either an increase in steady state concentration of the bound ADP by addition of ADP or a decrease in the rate of its release. Thus, the sigmoidal response caused by added ADP could be a result of increase in the steady state concentration of the bound ADP. Preincubation of the enzyme with Ca\(^{2+}\) ions, which were shown to slow down the release of ADP, increased the values of the Hill coefficient. Under each of these conditions, a larger Hill coefficient value was obtained as the reaction time was shortened so that only a minimal amount of the bound ADP was released following the onset of the reaction. Vanadate inhibited in a cooperative manner, probably by binding to the ADP site and forming a vanadyl-ADP intermediate. In preliminary experiments, we have shown a slow down in the rate of release of the exchangeable ADP in the presence of vanadate Ca\(^{2+}\) and ADP. Factors which increased the Hill coefficient values in the presence of ADP also affected the sigmoidity obtained in the presence of vanadate. Thus, the highest Hill coefficient value was obtained in the presence of both ADP and vanadate.

Determination of \(K_i\), for ADP as Modulator of Vanadate Inhibition—The modulation of the \(K_i\) for vanadate inhibition by ADP enabled the determination of the dissociation constant for the two inhibitory components. The \(K_i\) of the ADP for modulation of the inhibition of activity in the presence of vanadate was determined. As the experiments were conducted at constant CaCl\(_2\) concentration, a change in total ADP resulted also in changes in the concentrations of CaADP and free ADP. It was difficult to conclude from the analysis which of the three components of ADP was inhibitory, but it was possible to determine the \(K_i\) for each of them. CaADP, which had the lowest \(K_i\), could be the best candidate for the inhibition. The possibility that CaADP rather than free ADP enhanced the inhibition by vanadate was supported by the finding that free Ca\(^{2+}\), in the absence of added ADP, also increased the inhibition by vanadate. The \(K_i\) for the effect of free Ca\(^{2+}\) was 1.7 ± 0.2 \(\mu\)M, similar to the \(K_i\) for divalent ions which slow the release of the enzyme-bound ADP at site 1. It is suggested that the slowing of the release of ADP was due to binding of Ca\(^{2+}\) to the same site. Yet there was a more than 20-fold difference between the dissociation constant for the binding of Ca\(^{2+}\) to the enzyme-bound ADP and the binding of free Ca\(^{2+}\) to the enzyme in spite of the fact that the two species induced a decrease in the \(K_i\) for vanadate inhibition. The difference in the dissociation constants could be a result of the fact that free Ca\(^{2+}\) was bound to the enzyme-bound ADP, while free CaADP was bound to another site, with a higher dissociation constant. In the first case, the release of the bound CaADP was slowed down, thus increasing the concentration of the transition state conformation. In the second case, the binding of CaADP to a second site which was assumed to be in equilibrium with the first site increased the steady state concentration of the CaADP bound in site 1. A similar mechanism could explain the observed inhibition of the activity caused by preincubation of the enzyme with CaADP, which had a similar \(K_i\) (35). It is suggested that CaADP was bound to a second catalytic site. A similar \(K_i\) for Micrograms of ADP during photophosphorylation was determined for a suggested second catalytic site occupancy (36) while a much lower \(K_i\) for micrograms of ADP was determined for the first catalytic site (37, 38).

The \(K_i\) for Vanadate Inhibition—A marked decrease in the \(K_i\) for vanadate inhibition was observed under conditions which favored the increase in the concentration of the enzyme-bound ADP. This was achieved under the experimental conditions which induced a high Hill coefficient with respect to CaADP. Thus, preincubation of the enzyme in the presence of divalent metal ions, assay for a short time in the presence of CaADP, low substrate concentration, and free Ca\(^{2+}\) ions gave the lowest \(K_i\) for vanadate inhibition. It is suggested that under such conditions the concentration of the transition state of the enzyme was increased and favored the formation of a pentacovalent vanadyl-ADP by the enzyme-bound ADP and vanadate. This analogue of the transition state intermediate had a low dissociation constant and was only slowly released from the enzyme on addition of substrate, causing a strong inhibition. Yet the \(K_i\) obtained from direct binding measurements of vanadate to three interacting sites on the enzyme was much lower than \(K_i\) for inhibition, thus indicating that under steady state conditions only part of the enzyme molecules assumed transition state conformation. It can be argued further that the fact that vanadate, which tends to form a pentacovalent intermediate, was a most effective inhibitor when bound to the enzyme-bound ADP supports the suggestion that the ADP was bound tightly because the subunit assumed a transition state conformation.

Monovanadate Rather Than Di- or Tetravanadate Caused the Inhibition—Three modes of inhibition could be observed in enzymes which were inhibited by vanadate. Monovanadate inhibited by directly binding to the enzyme, by binding as an adduct substrate or a product, or by binding as one of the polymeric forms of vanadate (16). It was therefore important to verify which was the mode of vanadate inhibition in CF\(_1\). The use of \(^{31}\)NMR spectroscopy enabled the discrimination of the concentration of the monomeric and the various polymeric forms at a given experimental condition. Although almost complete inhibition of ATP\(_\text{ase}\) activity was observed at concentrations ranging from 0–0.1 \(\mu\)M total vanadate, there was a lag in the increase of the relative concentrations of di- and tetravanadate. If divanadate or tetravanadate were the inhibitors, a lag in the inhibition as a function of the total vanadate concentration would be expected. Yet, no such lag was observed. The linearity of the Dixon plot of the change in the \(K_i\) for inhibition by CaADP as a function of monovanadate concentration compared to deviation from linearity in the case of di- and tetravanadate also indicated that the former was the inhibitory species. Changes in the medium pH dramatically altered the relative concentrations of di- and tetravanadate, but showed no correlation between the extent of inhibition and the increase in the concentration of these forms of vanadate. There was, however, a good correlation between monovanadate concentration and the extent of inhibition of ATP\(_\text{ase}\) activity.

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