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Kinetics of Oxidative Phosphorylation in *Paracoccus denitrificans*. 2. Evidence for a Kinetic and Thermodynamic Modulation of F_oF_1-ATPase by the Activity of the Respiratory Chain†

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ABSTRACT: (1) The affinity of the F_oF_1-ATPase from *Paracoccus denitrificans* for ATP during NADH-driven oxidative phosphorylation has been analyzed under different conditions by examining the type and extent of product inhibition. (2) A limited collapse of the protonmotive force (Δp) due to partial uncoupling does not increase the affinity for ATP at the active site(s) of the enzyme; instead, a partial noncompetitive inhibition becomes apparent, compatible with the binding of ATP to a noncatalytic site (or sites) with high affinity. (3) In contrast, partial inhibition of the electron-transport chain increases the extent of pure competitive product inhibition and, therefore, the affinity for ATP at the active site(s). (4) The results are interpreted as indicative of a modulation of the rate of ATP release from the active site(s) of the F_oF_1-ATPase which is controlled by the activity of the electron-transport chain and not by Δp.

In the mechanism of ATP synthesis catalyzed by F_oF_1-ATPases,[1] the release of ATP from the active site(s) is considered to be the step with the largest demand for energy [e.g., Boyer et al. (1973) and Hackney et al. (1979)]. In agreement with this idea, the onset of respiration in beef heart submitochondrial particles was observed to reduce dramatically the affinity for ATP at the active site of the F_oF_1-ATPase (Penefsky, 1985). According to the simplest delocalized version

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[1] Abbreviations: Δp, protonmotive force or transmembrane electrochemical proton gradient expressed in millivolts; F_oF_1-ATPase, H^+-translocating ATPase type F_oF_1 (ATP-synthase; EC 3.6.1.3); F_1, soluble catalytic sector of F_oF_1-ATPase; P_i, inorganic phosphate; G6P, glucose 6-phosphate; HK, hexokinase; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone.

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of the chemiosmotic hypothesis, the energy transferred to this enzyme through H^+ translocation is stored in the protonmotive force, Δp (Mitchell, 1966); consequently, it can be expected that alterations in the size of Δp will have a significant effect on the kinetics of ATP release, independently of the way in which Δp has been modified.

It has been generally observed that, during net ATP synthesis, a limited collapse of Δp due to partial uncoupling produces significant increases in the apparent Michaelis constants for ADP and P_i [e.g., Vinkler (1981), Hatefi et al. (1982), and McCarthy and Ferguson (1983)], while limitation of the electron flow decreases these parameters [e.g., Kayalar et al. (1976), Vinkler (1981), Bickel-Sankötter and Strotmann (1981), and Yagi et al. (1984)]. Recently, Quick and Mills (1987, 1988) have defended the compatibility of these observations with a fully delocalized model of chemiosmotic energy coupling: this is based on the fact that the uncontrolled variation in Δp, which occurs when the concentrations of ADP or P_i are changed, represents a severe hindrance to the determination of mechanistically significant values for K_m^{ADP} and $K_m^{P_i}$; the apparent values obtained will therefore be an underestimation of the true Michaelis constants (Quick & Mills, 1987, 1988). To support such analysis, these authors used model simulations of photophosphorylation in chloroplasts which appeared to reproduce successfully the reported variations in ${}^{app}K_m^{ADP}$ and ${}^{app}K_m^{P_i}$ observed at low rates of electron transport.

It was shown in the preceding paper (Pérez & Ferguson, 1990) that, under the experimental conditions tested, the values of ${}^{app}K_m^{ADP}$ and ${}^{app}K_m^{P_i}$ measured for oxidative phosphorylation in *Paracoccus denitrificans* are the actual values of the real Michaelis constants, in disagreement with the views expressed by Quick and Mills (1987, 1988). This conclusion was based on the flux control coefficient found for the F_oF_1-ATPase (equal to 1), a result which is in accordance with previous reports for the mitochondrial enzyme (Herweijer et al., 1985; Petronilli et al., 1988). Any changes in the values of K_m^{ADP} and $K_m^{P_i}$ will therefore result from variations in one (or some) of the rate constants associated with the steps of the mechanism at the active site(s) of the F_oF_1-ATPase. A recent report by Bizouarn et al. (1989) seems to point in this direction. For the reasons explained above, changes in the rate of ATP release should be largely responsible for variations in the Michaelis constants for ADP and P_i. To date, this possibility, which was not addressed by Quick and Mills (1987, 1988), has escaped scrutiny.

The preceding paper reported studies of product inhibition during ATP synthesis catalyzed by membrane preparations from *P. denitrificans* (Pérez & Ferguson, 1990). These studies allowed the affinity for ATP at the active site(s) of the F_oF_1-ATPase during net phosphorylation to be determined. The same methods have been used here to monitor any changes in this affinity which occur when partial uncoupling or limitation of the electron flow takes place.

Materials and Methods

Methods used were essentially as described in the preceding paper. Titration of the enzyme hexokinase (HK), included in some of the reaction mixtures, was required in some cases. The specific activity of the HK solutions was determined spectrophotometrically with a coupled assay by measuring the increase of absorbance at 340 nm due to the reduction of $NADP^+$ with G6P, catalyzed by G6P dehydrogenase. In a total volume of 3 mL, the reaction mixtures contained 10 mM P_i/21 mM Tris (pH 7.3), 5 mM magnesium acetate, 1% (v/v) ethanol, 20 mM glucose, 1 mM $NADP^+$, 5 mM MgATP, and 0.5–2.0 units·mL^{-1} of G6P dehydrogenase, at 30 °C. $NADP^+$ reduction was started by the addition of varying concentrations of HK (3.5–14.0 μg of HK·mL^{-1}), and the increase of absorbance was recorded. Slopes were measured after steady-state was reached and provided the maximal rate associated with each concentration of HK, as the concentrations of glucose, ATP, and $NADP^+$ were manyfold higher than the corresponding Michaelis constants (Glaser & Brown, 1955; Robertson & Boyer, 1956). The time required to reach steady state was longer when the concentration of the coupling enzyme G6P dehydrogenase was reduced (Storer & Cornish-Bowden, 1974).

Table I. Effect of Partial Uncoupling and of Partial Inhibition of the Electron-Transport Chain on the Kinetics of Oxidative Phosphorylation of *P. denitrificans*

(A) ADP as Variable Substrate[a] experiment	V_{max}[b]	K_m^{ADP}[b]	V_{max}/K_m^{ADP}
(1) control	2610	9.0	290
+FCCP (1.0 μM)	940	21.0	45
(2) control	2100	12.0	175
+cyanide (15 μM)	360	2.2	164
+myxothiazol [0.21 μg·(mg of protein)$^{-1}$]	420	2.5	168
(3) control	1210	10.0	121
+rotenone [10.5 μg·(mg of protein)$^{-1}$]	520	4.6	113

(B) P_i as Variable Substrate[c] experiment	V_{max}[b]	$K_m^{P_i}$[b]	$V_{max}/K_m^{P_i}$
(1) control	1680	170	10.0
+FCCP (0.8 μM)	1170	750	1.6
(2) control	1640	170	9.6
+cyanide (7.5 μM)	760	75	10.1
+myxothiazol [0.18 μg·(mg of protein)$^{-1}$]	520	50	10.4

[a] The rate of phosphorylation was measured as in Figure 1 of the preceding paper (Pérez & Ferguson, 1990) except that, where indicated, uncoupler or inhibitor was added at least 3 min before respiration was started. The kinetic parameters were derived from the corresponding Hanes plots of [ADP]/v vs [ADP]. [b] The units used for V_{max} and K_m^{ADP} ($K_m^{P_i}$) are nmol of ATP·min^{-1}·(mg of protein)$^{-1}$ and μM, respectively. [c] The rate of phosphorylation was measured as in Figure 2 of the preceding paper (Pérez & Ferguson, 1990). The concentration of ADP used was 180 μM in all cases. As before, the kinetic parameters were obtained from the linearized plots of the corresponding progress curves.

Results

Effect of Partial Uncoupling and of Partial Inhibition of the Electron-Transport Chain on Kinetics of ATP Synthesis. The effect that (a) partial uncoupling or (b) partial inhibition of the electron-transport chain has on the kinetics of the ATP synthesis was first investigated by monitoring the changes in the values of V_{max}, K_m^{ADP}, and $K_m^{P_i}$ which take place upon addition of FCCP or inhibition of the respiratory chain (Table I). As observed before in this and other systems (see the introduction), while partial uncoupling decreases V_{max} and increases both K_m^{ADP} and $K_m^{P_i}$, therefore decreasing V_{max}/K_m^{ADP} and $V_{max}/K_m^{P_i}$, the effect of a partial inhibition of the electron-transport chain is to reduce V_{max} and both K_m^{ADP} and $K_m^{P_i}$, leaving V_{max}/K_m^{ADP} and $V_{max}/K_m^{P_i}$ largely unmodified (Table I).

As in Table I, when the values of K_m^{ADP} and $K_m^{P_i}$ are being determined, it is standard practice to maintain a saturating concentration of the second substrate. Here, nevertheless, it was also important to evaluate whether the changes observed in the Michaelis constants are due to real changes in the affinity of the enzyme for its substrate(s) or to alterations in other parameters of the catalytic cycle. For this reason, initial rates of phosphorylation were also measured in the presence

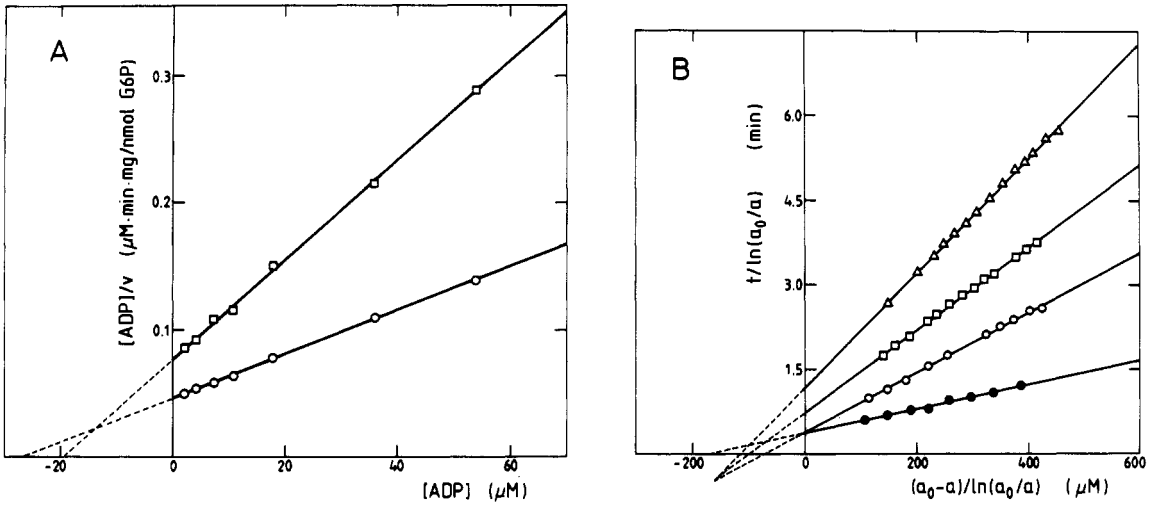

FIGURE 1: Effect of (A) partial uncoupling and (B) partial inhibition of the respiratory chain on the relative values of Michaelis and dissociation constants for ADP and P_i. (A) Initial rates of phosphorylation catalyzed by membrane vesicles (0.22 mg of protein·mL^{-1}) were measured in the presence of [^{32}P]P_i [10 (○) or 0.5 mM (□)] and a range of ADP concentrations, together with 1.6 μM FCCP. The data were plotted as Hanes plots of [ADP]/v vs [ADP]. For the reactions with 0.5 mM P_i (□), the production of [^{32}P]G6P was monitored by the withdrawal of aliquots at 20-s intervals for 4 min and remained linear throughout. (B) Progress curves were obtained for the formation of [^{32}P]G6P from 0.5 mM [^{32}P]P_i and ADP [3.6 (△), 7.2 (□), or 179 μM (○)], catalyzed by membranes vesicles (0.25 mg of protein·mL^{-1}) in the presence of 7.5 μM potassium cyanide. A control reaction with 179 μM ADP and no added cyanide was also obtained (●). The data were plotted according to $t/\ln(a_o/a)$ vs $(a_o - a)/\ln(a_o/a)$, where $a \equiv [P_i]$ [as in Figure 2 of Pérez and Ferguson (1990)].

of nonsaturating concentrations of the second substrate under conditions of partial uncoupling or of partial inhibition of the respiratory chain (Figure 1). It had been shown in the preceding paper that, in the absence of other effectors, the Michaelis constants for both ADP and P_i were identical with their respective dissociation constants, $K_m^{ADP} = K_i^{ADP}$ and $K_m^{P_i} = K_i^{P_i}$ [Figure 2 in Pérez and Ferguson (1990)]. As it is shown here, that is not the case when partial uncoupling occurs (Figure 1A): the increases of $K_m^{P_i}$ and K_m^{ADP} which take place when the uncoupler FCCP is added (Table I) are not followed by parallel increases of $K_i^{P_i}$ and K_i^{ADP}; the dissociation constants are now significantly smaller than the corresponding Michaelis ones, thus generating Hanes plots of lines with a common point above the x axis (Figure 1A shows the results obtained when ADP was the variable substrate, at fixed [P_i]; similar results, not shown, were obtained when P_i was the variable substrate, at fixed [ADP]). Not surprisingly, the opposite is found as a result of partial inhibition of the respiratory chain (Figure 1B): the decreases of K_m^{ADP} and $K_m^{P_i}$ are not followed either by similar decreases of K_i^{ADP} and $K_i^{P_i}$; the dissociation constants are significantly larger than their Michaelis counterparts, and the Hanes plots produce lines with a common point below the x axis (Figure 1B shows the results obtained when the respiratory chain was partially inhibited by cyanide; similar results, not shown, were obtained with other respiratory inhibitors, including rotenone and myxothiazol).

The results of Figure 1 indicate that the changes in the Michaelis constants produced by alterations of the coupling process are not due to similar changes in the real affinities for the substrates ADP and P_i, as previously thought [e.g., Hatefi et al. (1982)]. Instead, such changes will be forced by modifications of other steps in the catalytic mechanism of the F_oF_1-ATPase which occur after substrate binding (see Discussion).

Effect of Partial Uncoupling on the Type and Extent of Product Inhibition during Net ATP Synthesis. It was shown in the preceding paper that, during NADH-driven oxidative phosphorylation, the binding of ATP at the active site(s) of the F_oF_1-ATPase from *P. denitrificans* produces pure competitive product inhibition of the reaction of ATP synthesis, given by an inhibition constant of $K_i^{ATP} = 16 \pm 1$ μM [Table IV in Pérez and Ferguson, (1990)]. This inhibition constant is equivalent to the real dissociation constant for the binding of ATP; therefore, it provides a direct measure of the affinity of the enzyme for the nucleotide at the active site(s) during net phosphorylation.

In order to characterize the relationship between Δp and the affinity of the active site(s) for ATP, the effect that partial uncoupling produces on the extent of product inhibition was investigated. First, progress curves were obtained for the phosphorylation of a limited concentration of ADP (saturating [P_i]) in the absence of HK and glucose and in the presence of increasing concentrations of the potent uncoupler FCCP (Figure 2A). As in the preceding paper (Pérez & Ferguson, 1990), the reactions were analyzed by means of integrated forms of the Michaelis–Menten rate equation (Cornish-Bowden, 1981) and plotted according to

$$t/\ln(b_o/b) = {}^{app}K_m^{ADP}/{}^{app}V_{max} + (1/{}^{app}V_{max})[(b_o - b)/\ln(b_o/b)] \quad (1)$$

where $b \equiv$ [ADP], b_o symbolizes the initial concentration of ADP, and t is the time of reaction. As before, the apparent parameters ${}^{app}K_m^{ADP}$ and ${}^{app}V_{max}$ depend directly on the real parameters, K_m^{ADP} and V_{max}, and on the type and extent of product inhibition. As shown in Figure 2A, the effects of increasing concentrations of FCCP on the progress curves are to decrease progressively ${}^{app}V_{max}$ (the reciprocal of the slopes) and increase ${}^{app}K_m^{ADP}$ (the intercepts with the x axis). These results are similar to those obtained for K_m^{ADP} and V_{max} when initial rates are being measured (see Table I). They do not indicate any significant increases in the extent of competitive product inhibition and, consequently, in the affinity of the active site(s) for ATP.

The experiment of Figure 2A may be complicated by the decreased phosphorylation potential (ΔG_P), which is attained in the presence of FCCP [Figure 4 in Pérez and Ferguson (1990)], a consequence of the partial collapse of Δp. If smaller values of ΔG_P were to reduce the rates of phosphorylation due to thermodynamic restrictions, the plots of Figure 2A would be expected to deviate toward higher values of ${}^{app}K_m^{ADP}$ and ${}^{app}V_{max}$. For the following discussion it is important to note that, if that were the case, the plots of Figure 2A would

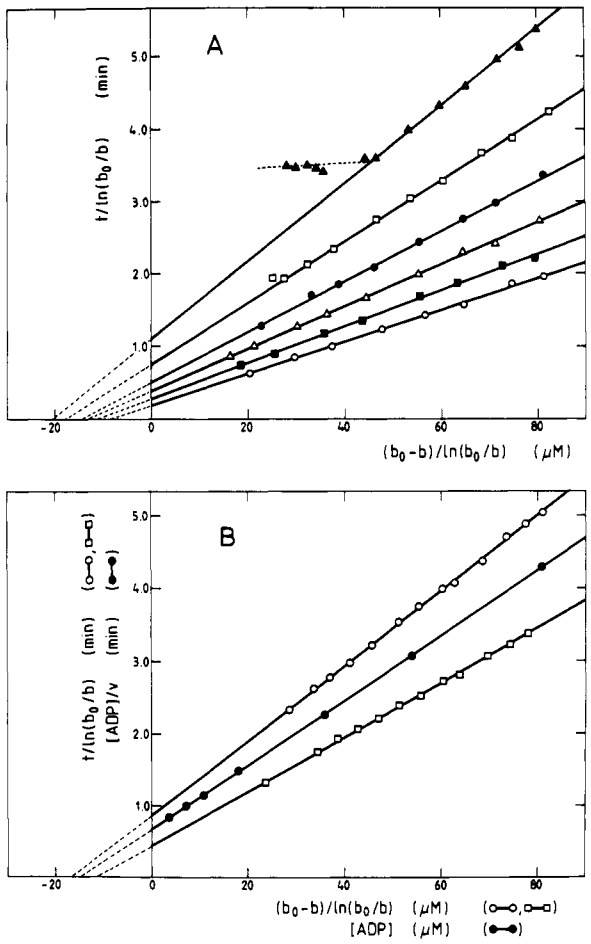

FIGURE 2: Characterization of the type and extent of product inhibition during partial uncoupling. (A) Progress curves of the formation of [γ-^{32}P]ATP from 10 mM [^{32}P]P_i and 94 μM ADP catalyzed by membrane vesicles (37.5 μg of protein·mL^{-1}) were obtained as before (Pérez & Ferguson, 1990), though in the presence of the following range of FCCP concentrations: 0 (○), 0.2 (■), 0.4 (△), 0.6 (●), 0.8 (□), and 1.0 μM (▲). The data from the progress curves were plotted according to eq 1 (see text). (B) The progress curves of the formation of [γ-^{32}P]ATP from 10 mM [^{32}P]P_i and 90 μM ADP catalyzed by membrane vesicles (18.2 μg of protein·mL^{-1}) in the presence (○) and absence (□) of 0.3 μM FCCP were obtained as in (A) and plotted according to eq 1. In the same experiment, initial rates of phosphorylation were also measured in the presence of HK and glucose, 10 mM [^{32}P]P_i, a range of ADP concentrations, and 0.3 μM FCCP (●); the data are represented as the corresponding Hanes plot of [ADP]/v vs [ADP]. The value of the kinetic parameters derived from the progress plot and from the initial rates obtained in the presence of FCCP are shown in Table II, as those corresponding to the experiment of Figure 4.

represent overestimates of the extent of competitive product inhibition and not underestimates. Note that the progress plot for the highest concentration of FCCP (1 μM in Figure 2A) suggests that deviations only occur as thermodynamic equilibrium is approached, but not before.

Further examination of product inhibition in the presence of FCCP was carried out by recording a progress plot like those of Figure 2A, together with a Hanes plot of initial rates obtained in the presence of HK/glucose and a range of ADP concentrations (Figure 2B). This experiment was analogous to a previous one shown in the preceding paper and performed in the absence of uncoupler [Figure 5 in Pérez and Ferguson (1990)]; an additional progress curve in the absence of FCCP was also included as a control (Figure 2B). The difference between $^{app}K_m^{ADP}$ (progress plot) and K_m^{ADP} (initial rates) in the presence of FCCP was as small as previously observed in the absence of uncoupler (Table II), confirming the results

Table II: Effect of Partial Uncoupling and Partial Inhibition of the Electron-Transport Chain on the Type and Extent of Product Inhibition during ATP Synthesis Catalyzed by Membrane Vesicles from *P. denitrificans*[a]

experiment[b]	V_{max}	$^{app}V_{max}$	K_m^{ADP}	$^{app}K_m^{ADP}$
(1) control[c]	1540	1540	10.0	11.7
(2) FCCP (0.3 μM) (Figure 2B)	1230	1050	15.3	16.3
(3) rotenone [23.8 μg·(mg of protein)$^{-1}$] (Figure 4B)	650	700	4.7	21.3
(4) cyanide (10 μM)	900	950	6.8	21.2
(5) myxothiazol [0.09 μg·(mg of protein)$^{-1}$]	630	690	5.9	22.6

[a] The kinetic parameters are presented following the convention of the preceding paper (Pérez & Ferguson, 1990); V_{max} and K_m^{ADP} are used to indicate the maximal rate and Michaelis constant derived from measures of initial rates of phosphorylation, while $^{app}V_{max}$ and $^{app}K_m^{ADP}$ are those derived from the progress curves. The units used for K_m^{ADP} ($^{app}K_m^{ADP}$) and V_{max} ($^{app}V_{max}$) are μM and nmol of ATP·min^{-1}·(mg of protein)$^{-1}$, respectively. For experiments 2–5, the progress curves carried out in the absence of uncoupler or inhibitor are not included for simplicity. [b] The figures where the original data are shown are indicated in parentheses. Experiments 4 and 5 (data not shown) followed the standard protocol of experiments 1–3. [c] The control experiment is taken from Figure 5 of the preceding paper (Pérez & Ferguson, 1990).

of the experiment shown in Figure 2A. This indicated that no substantial increase in the affinity of the active site(s) for ATP had occurred.

Unexpectedly, the same experiment showed that the presence of the uncoupler made $^{app}V_{max}$ (progress plot) clearly smaller than V_{max} (initial rates) (Table II). Such an effect of the ATP present during the progress of the reaction is not compatible with binding of the nucleotide at the active site(s) of the F_oF_1-ATPase (which should produce pure competitive product inhibition as reported earlier) but with binding of ATP at a different site to produce noncompetitive inhibition. As the application of eq 1 to the progress curves in the presence of FCCP (Figure 2) does not produce detectable deviations from linearity, the results strongly suggest the binding of ATP to at least one site with high affinity, which is saturated in the presence of low [ATP] but produces only partial noncompetitive inhibition.

To confirm that ATP binds with high affinity to a noncatalytic site which, as a consequence of partial uncoupling, can partially block the activity of the F_oF_1-ATPase, an additional experiment was devised. A decreasing range of HK concentrations allowed the accumulation of increasing but very low [ATP] during phosphorylation in the presence of FCCP (Figure 3). Controls in the absence of FCCP showed that the lowest [HK] used was still sufficient to maintain the rate of phosphorylation. However, when a high concentration of FCCP was used, an increasing inhibition of the reaction was observed, which was greater as [HK] was reduced. For the range of [HK] used, [ATP] in steady state could be estimated (Aflalo & Shavit, 1982), the value of the Michaelis constant of HK for ATP being taken as 100 μM (Glaser & Brown, 1955); for the lowest [HK] assayed, [ATP], was calculated to be only 6.5 μM, still insufficient to generate thermodynamic limitations to the reaction. At this point, the extent of inhibition (50%) seemed to be close to its maximal value, as expected from the near saturation of the binding site for ATP responsible for the effect.

Effect of Partial Inhibition of the Electron-Transport Chain on the Type and Extent of Product Inhibition during Net ATP Synthesis. The unexpected finding that partial uncoupling did not increase the affinity for ATP of the active site(s) of the F_oF_1-ATPase during net phosphorylation questioned the direct role of Δp in promoting the release of the nucleotide (see the introduction). An alternative factor which may also

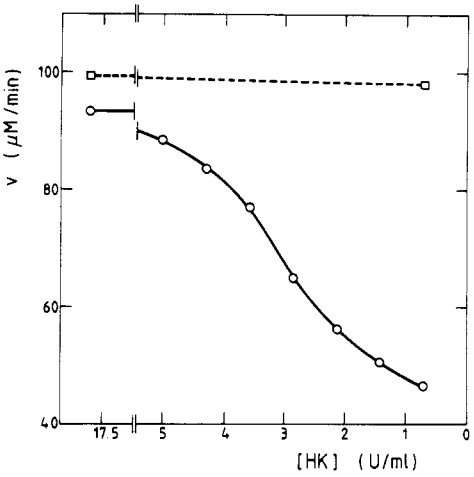

FIGURE 3: Inhibition by low concentrations of ATP during partial uncoupling. Initial rates of phosphorylation catalyzed by membrane vesicles (0.22 mg of protein·mL^{-1}) from *P. denitrificans* were measured in the presence of 10 mM [^{32}P]P$_i$, 135 μM ADP, 1.6 μM FCCP, 20 mM glucose, and a range of HK concentrations as indicated (○). The concentrations of HK (expressed in units·mL^{-1}, where 1 unit = 1 μmol of G6P·min^{-1}) were titrated as described under Materials and Methods. As a control, initial rates catalyzed by membrane vesicles (43 μg of protein·mL^{-1}) in the absence of FCCP were also measured in the presence of the highest and the lowest [HK] (□).

hamper ATP synthesis is a limitation of the rate of electron flow through the respiratory chain, and this was first examined by partially inhibiting the complex NADH dehydrogenase with rotenone. Progress curves were obtained for the phosphorylation of a limited amount of ADP (saturating [P$_i$]) in the absence of HK and glucose and in the presence of increasing concentrations of rotenone (Figure 4A). The reactions were analyzed by means of eq 1 as before. It was found that, as the extent of inhibition by rotenone increased, the apparent maximal rate $^{app}V_{max}$ was reduced, while the apparent Michaelis constant for ADP ($^{app}K_m^{ADP}$) and the ratio $^{app}K_m^{ADP}/^{app}V_{max}$ were increased (Figure 4A). These results were significantly different from those obtained by measuring initial rates (i.e., in the absence of any product inhibition) when rotenone decreased both V_{max} and K_m^{ADP}, leaving the ratio K_m^{ADP}/V_{max} largely unmodified (Table I). As shown in the preceding paper, the partial inhibition of the respiratory chain has only a small effect on the size of the phosphorylation potential ΔG_P [see Figure 4 in Pérez and Ferguson (1990)], so thermodynamic restrictions cannot account for the differences between progress curves and initial rates.

The results of Figure 4A suggest very strongly that the extent of product inhibition is much greater in the presence of rotenone. To confirm this observation, a progress curve was recorded and shown together with initial rates obtained in the presence of HK/glucose and a range of [ADP] (Figure 4B). As expected, $^{app}K_m^{ADP}$ was significantly greater than K_m^{ADP}, while $^{app}V_{max}$ was also slightly greater than V_{max} (Table II). This behavior is indicative of a large increase in the extent of pure competitive product inhibition and, therefore, in the affinity for ATP at the active site(s) (compared with experiment 1 in Table II).

Experiments similar to that of Figure 4B were also carried out with the respiratory chain partially blocked by cyanide, which inhibits the terminal oxidases (Ferguson, 1982), and with myxothiazol, a very potent inhibitor of the bc_1 complex (Thierbach & Reichenbach, 1983). The presence of both inhibitors reproduced very closely the results obtained with rotenone (Table II), indicating that the increase in the extent of product inhibition is independent of the way in which the

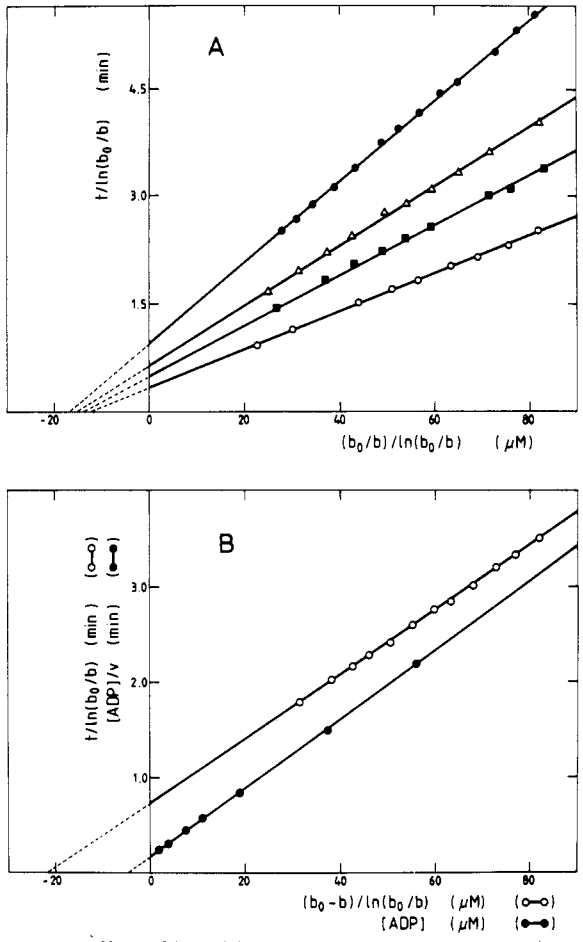

FIGURE 4: Effect of inhibition by rotenone on the type and extent of product inhibition. (A) Progress curves of the formation of [γ-^{32}P]ATP from 10 mM [^{32}P]P$_i$ and 93 μM ADP catalyzed by membrane vesicles (20 μg of protein·mL^{-1}) were obtained as in Figure 3, though in the presence of the following range of rotenone concentrations: 0 (○), 14.8 (■), 24.6 (△), and 37 μg·(mg of protein)$^{-1}$ (●). The data from the progress curves were plotted according to eq 1. (B) A progress curve of the formation of [γ-^{32}P]ATP from 10 mM [^{32}P]P$_i$ and 93 μM ADP catalyzed by membrane vesicles (42 μg of protein·mL^{-1}) in the presence of 23.8 μg of rotenone·(mg of protein)$^{-1}$ was obtained as in (A) and plotted according to eq 1 (○). In the same experiment, initial rates of phosphorylation were also measured in the presence of the same concentration of rotenone, HK/glucose, 10 mM [^{32}P]P$_i$, and a range of [ADP]; the data are represented as a Hanes plot of [ADP]/v vs [ADP] (●).

electron-transport chain is inhibited.

The results summarized in Table II allow the determination of the actual values of the inhibition constant for ATP (K_i^{ATP}) when the rate of electron flow is limited, by use of eq A13 ($^{app}V_{max}$), eq A14 ($^{app}K_m^{ADP}$), and Table IV from the preceding paper (Pérez & Ferguson, 1990). In the three cases (inhibition by rotenone, by cyanide, or by myxothiazol), eq A14 produces values of $K_i^{ATP} < 1$ μM, significantly smaller than the value obtained in the absence of inhibitors ($K_i^{ATP} = 16$ μM). The use of eq A13 yields values of K_i^{ATP} somewhat higher (around 40%) than those obtained with eq A14, indicating that the corresponding values of $^{app}V_{max}$ are slightly smaller than those compatible with the presence of only pure competitive product inhibition. This discrepancy suggests that a partial noncompetitive inhibition by ATP, like the one observed with partial uncoupling (Figures 2B and 3), could also be occurring.

Effect of Partial Inhibition of F_oF_1-ATPase on the Kinetics of ATP Synthesis upon Partial Inhibition of the Respiratory Chain. It was shown in the preceding paper that the reduction in the rate of respiration by rotenone did not affect the extent

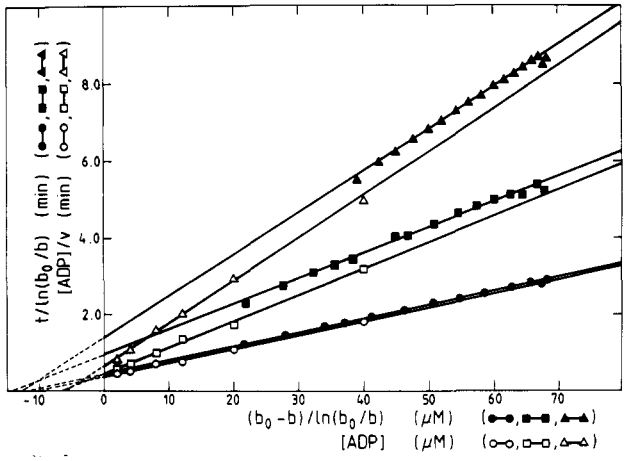

FIGURE 5: Effect of inhibition by venturicidin on the kinetics of ATP synthesis upon partial inhibition of the respiratory chain. Progress curves of the formation of [γ-^{32}P]ATP from 10 mM [^{32}P]P_i and 99 μM ADP catalyzed by membrane vesicles (12 μg of protein·mL^{-1}) were obtained as in Figure 3, though in the presence (■, ▲) or absence (●) of 62.5 μg of rotenone·(mg of protein)$^{-1}$. In one case (▲), venturicidin was present [41.7 μg·(mg of protein)$^{-1}$] together with rotenone. The data from the progress curves were plotted according to eq 1. In the same experiment, initial rates of phosphorylation were also measured in the presence of HK/glucose, 10 mM [^{32}P]P_i, and a range of [ADP], with (□, △), or without (○) added rotenone and venturicidin (△) as before; the data are represented as Hanes plots of [ADP]/v vs [ADP].

of inhibition by venturicidin of the rate of phosphorylation at saturating [ADP] and [P_i] [Figure 3B in Pérez and Ferguson (1990)]. From this observation, it was concluded that the F_oF_1-ATPase remained fully rate limiting, despite the significant decrease in the rate of electron transport. To further explore the nature of the change in the affinity for ATP upon inhibition of the respiratory chain, an experiment similar to those summarized in Table II was performed, where progress curves were recorded together with initial rates, in the presence of both rotenone and venturicidin (Figure 5): it was found that neither the decrease of K_m^{ADP} nor the increase of $^{app}K_m^{ADP}$ (due to a higher affinity for ATP), which were the result of the addition of rotenone alone, was affected by the concomitant presence of venturicidin, despite inhibition of the F_oF_1-ATPase and a significant decrease in the rate of phosphorylation (Figure 5).

As discussed in Appendix I of the preceding paper (Pérez & Ferguson, 1990), the lack of effect on the experimental value of K_m^{ADP} ($K_m^{P_i}$) of a partial inhibition of the F_oF_1-ATPase is indicative of the enzyme being fully rate limiting and the measured K_m a real value. The results of Figure 5 are therefore a confirmation of the previous conclusion, even when the rate of electron transport has been substantially reduced. They also show that, under the experimental conditions tested, the increase in the affinity for ATP, observed upon limitation of the electron flow, is not affected by the relative rate of ATP synthesis.

Discussion

As stated in the introduction, during ATP synthesis catalyzed by F_oF_1-ATPases the main requirement for energy lies in the release of product from the active site(s) of the enzyme [e.g., Boyer et al. (1973) and Penefsky (1985)]. Accordingly, the largest proportion of free energy (ΔG) required by the reaction will be used to decrease by manyfold the affinity for ATP. The transfer of energy is believed to occur through a concerted conformational change between catalytic sites, which is induced by H^+ translocation [e.g., Kayalar et al. (1977) and Gresser et al. (1982)]. According to the chemiosmotic hypothesis, Δp supplies this energy to the F_oF_1-ATPase.

The constant for ATP corresponding to pure competitive product inhibition of the phosphorylating reaction (K_i^{ATP}) is also the real dissociation constant for the binding of the nucleotide to the active site(s) (Pérez & Ferguson, 1990). For this reason, it is quite surprising that partial uncoupling does not significantly increase the value of this constant, despite the marked decrease in the rate of phosphorylation (Figure 2). This finding raises some questions about the actual role of Δp in the promotion of ATP release, mainly because limitations in the rate of electron flow produce the predicted reductions in the value of K_i^{ATP} (Figures 4 and 5; Table II). It can be calculated from Figure 4 and experiments summarized in Table II that a decrease of around 50% in the maximal rate of phosphorylation due to partial inhibition of the electron-transport chain is linked to a 20-fold decrease in the value of K_i^{ATP}; if Δp is the only factor modulating the affinity for ATP, partial uncoupling should have produced similar changes in K_i^{ATP}, contrary to the experimental results (Figure 2).

Previous findings related to the promotion of ATP release from the active site(s) of the F_oF_1-ATPase during net ATP synthesis do not, in fact, contradict the observations reported here. In chloroplasts, limitation of the rate of electron flow by decreasing light intensity during photophosphorylation produces a marked increase in the number of reversals (ADP + P_i $\rightleftharpoons$ ATP + H_2O) at the active site prior to product dissociation (Hackney et al., 1979; Stroop & Boyer, 1987). This is compatible with a significant decrease in the rate of ATP release, relative to the rate of interconversion. Also in chloroplasts, decreasing light intensity has been found to increase the intermediate ATP $\rightleftharpoons$ H_2O oxygen exchange accompanying ATP synthesis (Spencer & Wimmer, 1985), which represents a similar observation. In the same study, however, no effect of partial uncoupling could be found on the exchange reaction (Spencer & Wimmer, 1985). This discrepancy resembles very closely the different effects of electron-flow limitation and partial uncoupling on the affinity for ATP, presented in this paper.

The preceding paper dealt with the mechanistic interpretation of the Michaelis constants for ADP and P_i determined for ATP synthesis (Pérez & Ferguson, 1990). It was shown there that these parameters are real kinetic constants of the F_oF_1-ATPase. Therefore, any changes in their values will be the result of modifications in at least one of the intrinsic rate constants of the enzyme; as discussed before (see the introduction), the rate of ATP release should be a main candidate to experience these variations. To facilitate the present analysis, the steps of the reaction of ATP synthesis can be written as

$$\text{Enz} + \text{S} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \text{Enz·S} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} \text{Enz·P} \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} \text{Enz} + \text{P} \quad (2)$$

where S ≡ ADP + P_i, P ≡ ATP, $k_{\pm 1,2,3}$ are the rate constants associated with the different steps, and Enz represents the F_oF_1-ATPase. With eq 2, the simplification to a "monosubstrate" enzyme can be used to derive, from a steady-state approach, the dependence of the kinetic parameters of the reaction on the different rate constants (Cornish-Bowden, 1981):

$$V_{max} = \frac{k_{+2}k_{+3}e_o}{k_{-2} + k_{+2} + k_{+3}} \quad (3)$$

$$K_m^S = \frac{k_{-1}k_{-2} + k_{-1}k_{+3} + k_{+2}k_{+3}}{k_{+1}(k_{-2} + k_{+2} + k_{+3})} \quad (4)$$

$$K_i^S = k_{-1}/k_{+1} \quad (5)$$

$$K_i^P = k_{+3}/k_{-3} \quad (6)$$

and also

$$K_m^S/V_{max} = \frac{k_{-1}k_{-2} + k_{+3}(k_{-1} + k_{+2})}{k_{+1}k_{+2}k_{+3}e_o} \quad (7)$$

V_{max} represents the maximal rate of phosphorylation, K_m^S is the Michaelis constant for the substrate, K_i^S is the dissociation constant of the substrate (its "inhibition" constant, when dealing with a two-substrate enzyme), and K_i^P is the dissociation constant of the product (also the constant for pure competitive product inhibition); e_o represents the total concentration of active enzyme capable of catalyzing the reaction (see below). Although eqs 2–7 are not strictly applicable to the F_oF_1-ATPase, they are useful to illustrate how changes in the rate of product release (k_{+3}) will affect the value of the different kinetic parameters.

As previously known, and confirmed in this paper for *P. denitrificans* (Table I), partial uncoupling increases the value of the apparent (=real) Michaelis constants for ADP and P_i, while limitation in the rate of electron flow has the opposite effect (see the introduction). From eqs 2–7, an analysis of these changes, together with the changes in product inhibition reported in this paper, can be based on modifications of k_{+3} as follows:

(i) Limitation of the rate of electron flow significantly reduces the value of k_{+3}. Such a reduction has a direct effect on K_m^S (eq 4) and V_{max} (eq 3) and decreases in parallel the value of both parameters.

(ii) The ratio K_m^S/V_{max} (eq 7) is much less sensitive to changes in k_{+3} and remains largely unmodified [Table I; also, e.g., Bickel-Sankötter and Strotmann (1981)], particularly if $k_{-2} < k_{+3}$.

(iii) By reduction of k_{+3}, the value of K_i^P (eq 6) is also decreased, so that extent of pure competitive product inhibition is increased (Figure 4 and Table II).

(iv) The affinity for the substrate(s), as given by K_i^S (eq 5), does not depend on k_{+3} and, therefore, remains unchanged. In the preceding paper it was shown that, for vesicles from *P. denitrificans*, K_m^S and K_i^S are identical when neither partial uncoupling nor limitation of the rate of electron flow takes place [Figure 2 of Pérez and Ferguson (1990)]. Therefore, the reduction of k_{+3} due to partial inhibition of the electron-transport chain would be expected to result in $K_m^S < K_i^S$, as observed (Figure 1B).

(v) Partial uncoupling not only produces a limited collapse of Δp but also induces a large increase in the rate of electron flow. For this reason, accepting that the activity of the electron-transport chain modulates k_{+3}, it can be speculated that a higher rate of ATP release will be generated under these conditions. In that case, greater values of K_m^S may be expected, thus making $K_m^S > K_i^S$ (Figure 1A).

(vi) If partial uncoupling accelerates the rate of ATP release, then the extent of competitive product inhibition should not be increased (Figure 2) but may actually be reduced; no results in support of this last proposal are presented here, because the partial collapse of Δp also reduces the size of ΔG_P, therefore hindering product inhibition studies in the presence of added ATP.

(vii) Provided the F_oF_1-ATPase behaves as a fully rate limiting enzyme for the phosphorylating reaction, a partial inhibition of the F_oF_1-ATPase itself will not affect k_{+3}; the lack of effect of venturicidin on K_m^{ADP} and $K_m^{P_i}$ is compatible with such a conclusion (Pérez & Ferguson, 1990). In the absence of any other effectors, this inhibitor is also without effect on the extent of product inhibition (not shown). The results of Figure 5 support the same interpretation even under conditions of marked reduction in the rate of electron flow.

The analysis described shows how modulation of k_{+3} by the relative rate of electron flow can account for variations in the kinetic parameters associated with ATP synthesis under various conditions. It is important to mention that the experimental findings reported in this paper do not provide any information about possible changes in the rates of interconversion at the active site (k_{+2} and k_{-2} in eq 2) which may well occur in parallel to changes in k_{+3}, as exchange data reported by Stroop and Boyer (1987) strongly suggest. Nevertheless, examination of eqs 2–7 shows that such putative changes are readily compatible with the proposed modulation of k_{+3} and the analysis presented.

Although the putative decrease in the extent of competitive product inhibition during partial uncoupling was not investigated here for the reason already given, results reported by Aflalo and Shavit (1983) strongly suggest this possibility. These authors found that hypotonically treated chloroplasts catalyze a reaction of phosphorylation which is characterized by higher values of K_m^{ADP} and $K_m^{P_i}$, compared to nontreated chloroplasts, without changes in the maximal rate V_{max}; the difference is probably due to enhanced diffusion of ligands in the microenvironment of the F_oF_1-ATPase after the hypotonic shock (Aflalo & Shavit, 1983). It was also found that, while the use of nonsaturating light intensities had the effect of enhancing the differences between treated and nontreated chloroplasts, the presence of uncouplers diminished these differences, or even negated them. To explain this surprising finding, Aflalo and Shavit (1983) proposed that uncouplers have the effect of facilitating the free diffusion of ligands. However, examination of eq 4 provides an alternative explanation; the direct dependence of K_m^S on k_{+3} means that the proposed acceleration of product release due to partial uncoupling will counteract the effect of hypotonic treatment on K_m^{ADP} and $K_m^{P_i}$, while limitation of the rate of electron flow will enhance it, in agreement with the finding (Aflalo & Shavit, 1983).

Equation 3 also shows that, if the value of k_{+3} is increased by partial uncoupling, V_{max} will also be increased; obviously, this is not the experimental finding (e.g., Table I and Figure 2). Nevertheless, eq 3 defines the value of V_{max} only for the F_oF_1-ATPase complexes capable of catalysis and does not account for the effect of Δp on the activation of the enzyme, particularly well established in chloroplasts [e.g., Gräber et al. (1977), Mills and Mitchell (1984), and Junesch and Gräber (1985)]; the limited collapse of Δp due to partial uncoupling does reduce the number of active complexes and, therefore, the apparent value of V_{max}, despite any increase of k_{+3}.

The results of Figures 2B and 3 show that, in addition to the effects of partial uncoupling on Michaelis constants, maximal rates, and competitive product inhibition already discussed, a partial noncompetitive product inhibition also becomes apparent. This inhibition is compatible with the binding of ATP to at least one noncatalytic site with high affinity, which resembles a similar binding of ATP previously observed in chloroplasts (Aflalo & Shavit, 1982). If both findings correspond to the same phenomenon, the fact that ATP bound to a noncatalytic site (or sites) reported here is trapped by hexokinase (Figures 2B and 3) implies that the diffusion barriers found in chloroplasts (Aflalo & Shavit, 1982, 1983) are not present in membrane vesicles from *P. denitrificans*. The role of this binding site or sites, regulatory or not, requires further investigation. Recently, Xue and Boyer (1989) reported that the binding of ATP to noncatalytic sites of

chloroplast F_1 has a strong effect on the hydrolytic activity of the enzyme. The results presented here suggest that the effect on catalysis of the ATP bound to these sites may depend largely on the size of Δp.

Recently, Matsuno-Yagi and Hatefi (1989) reported that the increase in the values of K_m^{ADP} and $K_m^{P_i}$ for oxidative phosphorylation by beef heart submitochondrial particles, which is produced by lipophilic weak acid uncouplers such as FCCP, is not found when partial uncoupling is produced by electrogenic ionophores such as gramicidin D or valinomycin plus nigericin; it was proposed that lipophilic weak acid uncouplers bind to the F_oF_1-ATPase directly and increase intrinsic uncoupling within the enzyme. In the study reported in this paper, only FCCP was used to induce partial uncoupling and a concomitant reduction of Δp. In contrast to the results of Matsuno-Yagi and Hatefi (1989), the increased values of K_m^{ADP} and $K_m^{P_i}$ produced by partial uncoupling with FCCP (Table I) are found to be a common result of the action of a diversity of uncouplers: valinomycin/K^+/nigericin in *P. denitrificans* (McCarthy & Ferguson, 1983) and nigericin/K^+ (Vinkler, 1981; Quick & Mills, 1987, 1988), ammonium chloride (Vinkler, 1981), or methylammonium chloride (Loehr et al., 1985) in chloroplasts, among other examples. Therefore, as it is highly unlikely that all these compounds binds to the F_oF_1-ATPase, it can be concluded that the increases of K_m^{ADP} and $K_m^{P_i}$ are a genuine consequence of partial uncoupling. In this sense, the effect of gramicidin on the kinetics of ATP synthesis must be regarded with caution, because of its unusual uncoupling properties recently reported (Luvisetto & Azzone, 1989a,b; Rottenberg & Koeppe, 1989a,b).

The effects of partial uncoupling and limitation of the rate of electron flow on the extent of pure competitive product inhibition are more puzzling than the proposed modulation of the rate of product release (k_{+3}), because the actual affinity for ATP at the active site(s) of the F_oF_1-ATPase has both a kinetic and a thermodynamic significance. The kinetic implication is that Δp is not the only factor which controls and modulates ATP synthesis. The concomitant thermodynamic implication is that Δp is not the only source of energy either. Therefore, a simple delocalized model of chemiosmotic energy transduction is not sufficient to account for these observations. The role that product release plays in the kinetics of oxidative phosphorylation and photophosphorylation also shows the limitations of the model of Quick and Mills (1987, 1988).

It is important to appreciate that the interpretation of the behavior of the F_oF_1-ATPase presented in this and the accompanying paper (Pérez & Ferguson, 1990) is based on control analysis, reinforced by the thermodynamic implications of the studies of product inhibition reported here. This approach has led to the conclusion that the variations in the kinetic and thermodynamic parameters of the F_oF_1-ATPase cannot be rationalized on the basis that Δp is the sole factor connecting the oxidation reactions of the respiratory chain to the response of the F_oF_1-ATPase. Even under conditions of moderate inhibition of the respiratory chain, truly mechanistic values of K_m^{ADP} and $K_m^{P_i}$ were obtained by a conventional K_m analysis, as established in Appendix I of Pérez and Ferguson (1990). This is valid irrespective of any differences in the values of Δp that may be associated with different concentrations of the substrates for the F_oF_1-ATPase (Quick & Mills, 1987, 1988). However, the conclusion reached should not be seen as advocating an entirely new coupling mechanism but rather as an indication of an additional feature to be incorporated into the current chemiosmotic framework. The need for it could only be refuted on the basis of an acceptable alternative interpretation of the data presented here. The observations presented in this paper do not in themselves give any insight into the nature of such a feature.

It is beyond dispute that artificial proton gradients Δp are capable of catalyzing ATP synthesis at high rates and, therefore, of promoting effectively the release of ATP [e.g., Thayer & Hinkle (1975), Junnesch and Gräber (1985), and Schmidt and Gräber (1985)]. Considering that the affinity for ATP at the active site(s) must be changed by a factor of 10^7 (transition from 10^{-12} to 10^{-5} M; Pérez & Ferguson, 1990), it is reasonable to assume that during electron transport driven ATP synthesis Δp also provides the largest share of free energy to the enzyme. However, an additional transfer of energy from the electron-transport chain to the F_oF_1-ATPase appears to occur, completing the coupling process. Quick and Mills (1987, 1988) found that partial inhibition of the F_oF_1-ATPase increased the values of K_m^{ADP} and $K_m^{P_i}$ for photophosphorylation in chloroplasts at low rates of electron transport, observations which are contrary to those reported here for inhibition with venturicidin in *P. denitrificans* (Figure 5; also Table I in the preceding paper): this discrepancy suggests that the additional coupling proposed between the electron-transport chain and the F_oF_1-ATPase does not occur below a given rate of electron flow, setting an upper limit on a completely delocalized mechanism. The nature of this supplementary energy transfer warrants further investigation.

Registry No. ATP, 56-65-5; ADP, 58-64-0; P_i, 14265-44-2; ATPase, 9000-83-3; ATP synthase, 37205-63-3.

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^{17}O, ^{1}H, and ^{2}H Electron Nuclear Double Resonance Characterization of Solvent, Substrate, and Inhibitor Binding to the $[4Fe\text{-}4S]^{+}$ Cluster of Aconitase†

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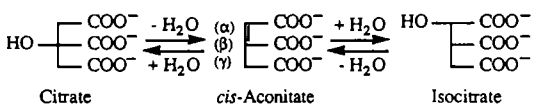
ABSTRACT: ^{17}O electron nuclear double resonance (ENDOR) studies at X-band (9-GHz) and Q-band (35-GHz) microwave frequencies reveal that the $[4Fe\text{-}4S]^{+}$ cluster of substrate-free aconitase [citrate (isocitrate) hydro-lyase, EC 4.2.1.3] binds solvent, H_xO (x = 1, 2). Previous ^{17}O ENDOR studies [Telser et al. (1986) *J. Biol. Chem. 261*, 4840-4846] had disclosed that $H_x{}^{17}O$ binds to the enzyme–substrate complex and also to complexes of enzyme with the substrate analogues *trans*-aconitate and nitroisocitrate (1-hydroxy-2-nitro-1,3-propanedicarboxylate). We have used ^{1}H and ^{2}H ENDOR to characterize these solvent species. We propose that the fourth ligand of Fe_a in substrate-free enzyme is a hydroxyl ion from the solvent; upon binding of substrate or substrate analogues at this Fe_a site, the solvent species becomes protonated to form a water molecule. Previous ^{17}O and ^{13}C ENDOR studies [Kennedy et al. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8854-8858] showed that only a single carboxyl, at C-2 of the propane backbone of *cis*-aconitate or at C-1 of the inhibitor nitroisocitrate, coordinates to the cluster. Together, these results imply that enzyme-catalyzed interconversion of citrate and isocitrate does not involve displacement of an endogenous fourth ligand, but rather addition of the anionic carboxylate ligand and a change in protonation state of a solvent species bound to Fe_a. We further report the ^{17}O hyperfine tensor parameters of the C-2 carboxyl oxygen of substrate bound to the cluster as determined by the field dependence of the ^{17}O ENDOR signals. ^{17}O ENDOR studies also show that the carboxyl group of the inhibitor *trans*-aconitate binds similarly to that of substrate.

The enzyme aconitase [citrate (isocitrate) hydro-lyase, EC 4.2.1.3] catalyzes the stereospecific interconversion of citrate and isocitrate via the dehydrated intermediate *cis*-aconitate.



The active site contains a diamagnetic $[4Fe\text{-}4S]^{2+}$ cluster which can be reduced to give the paramagnetic $[4Fe\text{-}4S]^{+}$ ($S = {}^{1}/_{2}$) rhombic EPR state ($g_{1,2,3}$ = 2.06, 1.93, 1.86) that binds substrate strongly with 30% retention of activity (Emptage et al., 1983a). Mössbauer spectroscopy shows that a single labile iron site, Fe_a, changes its coordination number upon addition of substrate to enzyme in either oxidation state (Kent et al., 1985). Pronounced shifts in the g value of the $[4Fe\text{-}4S]^{+}$ species are observed by EPR upon binding of the substrate and the substrate analogues *trans*-aconitate and nitroisocitrate. With bound substrate the paramagnetic form of the enzyme has the g values 2.04, 1.85, 1.78; with bound nitroisocitrate and with *trans*-aconitate the g values are 2.04, 1.87, 1.77 and

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