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Catalytic Sites of *Escherichia coli* F_1-ATPase. Characterization of Unisite Catalysis at Varied pH†

Marwan K. Al-Shawi and Alan E. Senior*
Department of Biochemistry, University of Rochester, School of Medicine and Dentistry, Rochester, New York 14642
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ABSTRACT: Using manual rapid-mixing procedures in which small, equal volumes of *Escherichia coli* F_1-ATPase and [γ-^{32}P]ATP were combined at final concentrations of 2 and 0.2 μM, respectively (i.e., unisite catalysis conditions), it was shown that ≥66% of the ^{32}P became bound to the enzyme, with the ratio of bound ATP/bound P_i equal to 0.4 and the rate of dissociation of bound [^{32}P]P_i equal to 3.5×10^{-3} s^{-1}, similar to previously published values. Azide is known to inhibit cooperative but not unisite catalysis in F_1-ATPase [Noumi, T., Maeda, M., & Futai, M. (1987) *FEBS Lett. 213*, 381-384]. In the presence of 1 mM sodium azide, 99% of the ^{32}P became bound to the enzyme, with the ratio of bound ATP/bound P_i being 0.57. These experiments demonstrated that when conditions are used which minimize cooperative catalysis, most or all of the F_1 molecules bind substoichiometric ATP tightly, hydrolyze it with retention of bound ATP and P_i, and release the products slowly. The data justify the validity of previously published rate constants for unisite catalysis. Unisite catalysis in *E. coli* F_1-ATPase was studied at varied pH from 5.5 to 9.5 using buffers devoid of phosphate. Rate constants for ATP binding/release, ATP hydrolysis/resynthesis, P_i release, and ADP binding/release were measured; the P_i binding rate constant was inferred from the ΔG for ATP hydrolysis. ATP binding was pH-independent; ATP release accelerated at higher pH. The highest K_a^{ATP} (4.4×10^9 M^{-1}) was seen at physiological pH 7.5. ATP hydrolysis and resynthesis were pH-independent, and the equilibrium constant for the cleavage/condensation reaction was around 2 at all pH values, showing catalysis occurred in a sequestered environment. P_i release was pH-independent, but P_i binding was drastically slowed at high pH. $K_d^{P_i}$, which was around 1 M at pH 5.5-7.5, reached 1.3×10^6 M at pH 9.5. ADP release was pH-independent; ADP binding was somewhat pH-sensitive, such that K_d^{ADP} decreased steadily from 42 μM at pH 5.5 to 430 nM at pH 9.5. The data confirm the view that, during normal oxidative phosphorylation, energy input from $\Delta\mu_{H^+}$ is required for P_i binding and ATP release. The data also support the idea that two major enzyme conformations are involved in unisite catalysis and that an ionic interaction influences binding of P_i and release of ATP in one conformation. In contrast to unisite ATP hydrolysis, multisite ATP hydrolysis was pH-dependent in the range pH 5.0-9.5, showing that the rate enhancements deriving from cooperative intersubunit interactions involve ionizable groups on the protein.

An important impetus for recent work on the mechanism of ATP synthesis by oxidative phosphorylation and photophosphorylation derived from the discovery of "unisite catalysis" in soluble F_1-ATPase from bovine heart mitochondria (Grubmeyer & Penefsky, 1981a,b; Grubmeyer et al., 1982). Unisite catalysis occurs when substrate ATP binds to a single site on F_1 and is characterized by (a) very high affinity binding of ATP, (b) reversible ($K_{eq} \sim 1$) hydrolysis and resynthesis of bound ATP to and from bound ADP and P_i, and (c) slow release of the products P_i and ADP. "Multisite catalysis", corresponding to V_{max} rates, occurs when two or three catalytic sites on F_1 are occupied by substrate and involves a large, positively cooperative acceleration of chemical catalysis and product-release rates (Cross et al., 1982). In more recent studies, Penefsky (1985a,b) demonstrated that unisite catalysis by bovine heart ATP synthase in membranes has similar characteristics to that of soluble F_1, and in response to questions regarding the significance of unisite catalysis in mitochondrial F_1 (Bullough et al., 1987), Penefsky (1988) and Cunningham and Cross (1988) demonstrated that unisite catalysis and its "promotion" to a multisite rate are a reflection of activity at a normal catalytic site. Unisite catalysis has been confirmed in F_1-ATPases from other sources including *Escherichia coli* (Wise et al., 1984) and yeast (Mueller, 1990), and Penefsky and Cross (1991) have recently reviewed the field. A scheme for unisite catalysis showing each of the four steps is

$$F_1 + ATP \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} F_1{<}ATP \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} F_1{<}ADP{\cdot}P_i \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}}$$
$$F_1{<}ADP + P_i \underset{k_{-4}}{\overset{k_{+4}}{\rightleftharpoons}} F_1 + ADP$$

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The significance of the discovery of unisite catalysis and of determination of its associated rate and equilibrium constants is severalfold. (a) It complements, supports, and extends the "binding change" model for ATP synthesis (Boyer, 1989). (b) It allows researchers to simplify the complex problem of studying F_1-ATPases by taking measurements under conditions where only a single catalytic site is operating and where the strong positive catalytic cooperativity is absent. (c) It allows thermodynamic analysis of the catalytic pathway, yielding insights into the mechanism (Al-Shawi & Senior, 1988; Al-Shawi et al., 1990a). Mutational analysis of unisite catalysis, introduced by Duncan and Senior (1985) in the *E. coli* enzyme, has proved to be a valuable approach to defining both the location and the action of the F_1-ATPase catalytic nucleotide-binding domain. From the data reported thus far, it has been suggested that the catalytic nucleotide-binding domain, located primarily or entirely on the β-subunit of F_1, and encompassing residues β137–335 approximately, provides a very high affinity, hydrophobic binding surface which stereochemically constrains and polarizes the substrates ATP or ADP·P_i, such that the chemical condensation reaction proceeds in a largely spontaneous, reversible fashion (Al-Shawi et al., 1990a). We surmise that a relatively large number of hydrogen-bonding and electrostatic interactions between the catalytic sites and substrates is involved, and we have demonstrated that conditions for normal catalysis, at least in *E. coli* enzyme, are not present in isolated α-subunit, isolated β-subunit, or $\alpha\beta$-subunit oligomer, but are realized in $\alpha_3\beta_3\gamma$-subunit oligomer (Al-Shawi et al., 1990b).

Here we extend our studies of unisite catalysis in soluble *E. coli* F_1-ATPase in several ways. First, we have demonstrated that when manual rapid-mixing techniques involving small, equal volumes of F_1 and substoichiometric radioactive ATP are used, with a final F_1/ATP concentration ratio of 10, the majority of the ATP is hydrolyzed by the unisite pathway, and we show that when cooperative catalysis is inhibited by use of sodium azide, all of the ATP is hydrolyzed in this manner. This was done to demonstrate that *E. coli* F_1 does not show heterogeneous hydrolysis of substoichiometric ATP [cf. Muneyuki et al. (1991)]. Second, we have determined unisite catalysis rate and equilibrium constants at varied pH. Multisite catalysis is known from previous work to be pH-dependent, with an optimum at ~pH 9, whereas at pH 5 and below there is low activity. It was therefore of interest to find how unisite catalysis varied in this range of pH, particularly because unisite rate constants are determined by pre-steady-state kinetic techniques in which F_1 itself is a reactant; thus, variations in constants and observed pK_a values refer directly to the reaction steps being measured, rather than to a combination of rate constants as in multisite catalysis. Third, a further point of interest was to study the dependence of *E. coli* F_1-ATPase unisite catalysis on exogenous P_i. Penefsky and Grubmeyer (1984) found that in bovine mitochondrial F_1, unisite catalysis was activated markedly by P_i. While the physiological significance of this finding is unclear since P_i would always be present in vivo, it was nevertheless of importance for comparative purposes to determine whether *E. coli* enzyme showed similar behavior.

Materials and Methods

Purification of Soiuble F_1-ATPase. Strain SWM1 (Rao et al., 1988) was the source. After growth of cells, all steps were at 4 °C. Membrane vesicles were prepared according to the procedure of Senior et al. (1979a). F_1 was released from membranes and precipitated with poly(ethylene glycol) as in Senior et al. (1979b) and then redissolved in "buffer A" consisting of pH 7.4 Tris–sulfate (50 mM), glycerol (10% v/v), $MgCl_2$ (2 mM), ATP (1 mM), dithiothreitol (DTT)[1] (1 mM), and 6-aminohexanoic acid (40 mM). Phenylmethanesulfonyl fluoride (1 mM) was added also at this stage. The enzyme was adsorbed on DEAE-Sepharose Cl-6B equilibrated in buffer A, pH 7.4, and the column was washed with buffer A plus 40 mM Na_2SO_4, pH 7.4. Then the enzyme was eluted with buffer A, pH 7.4, plus 100 mM Na_2SO_4, collected in fractions, and concentrated in an Amicon cell with an XM50 membrane. Concentrated enzyme was applied to a Sephacryl-S300 column equilibrated in buffer A, pH 7.0, plus 40 mM Na_2SO_4, peak fractions were concentrated as above, and the enzyme was stored in 100-μL aliquots (≥10 mg/mL) at −70 °C.

Buffers for Unisite Catalysis Assays. The following buffers were used: *pH 9.5*, 50 mM Tris–OH, 50 mM CHES, 4.5 mM K_2SO_4, and 0.5 mM $MgSO_4$, adjusted to pH with KOH; *pH 8.5*, 50 mM Tris–OH, 10 mM CAPS, 4.5 mM H_2SO_4, 9 mM KOH, and 0.5 mM $MgSO_4$; *pH 7.5*, 50 mM Tris–OH, 50 mM MOPS, 4.5 mM K_2SO_4, and 0.5 mM $MgSO_4$, adjusted to pH with H_2SO_4; *pH 6.5*, 50 mM Tris–OH, 50 mM MES, 4.5 mM K_2SO_4, and 0.5 mM $MgSO_4$, adjusted to pH with H_2SO_4; *pH 5.5*, 50 mM Tris–OH, 50 mM MES, 4.5 mM K_2SO_4, and 0.5 mM $MgSO_4$, adjusted to pH with H_2SO_4. All pH values were measured at 23 °C, using a Sigma glass calomel combination electrode.

Equilibration of F_1 with Buffers. One hundred microliters of F_1 was thawed and passed through 1-mL centrifuge columns (Penefsky, 1977) preequilibrated in the appropriate buffer at 23 °C. After protein assay (Bradford, 1976), the F_1 concentration was adjusted by addition of buffer.

Assays of the Rate Constants of Unisite Catalysis. All assays were done at 23 °C.

(1) ATP Binding (k_{+1}), Bound ATP Hydrolysis (k_{+2}), and ATP Resynthesis (k_{-2}). These constants were calculated from "acid-quench/cold-chase" experiments as described by Penefsky (1986). Equal volumes (12.5 μL) of F_1 and [γ-^{32}P]ATP were mixed rapidly using bent-tipped gas-tight microsyringes (Ray & Long, 1976) in a vortexed microfuge tube such that the final concentrations were 2 μM F_1/0.2 μM ATP. For the "cold-chase" experiments, at selected times (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 60, 150, and 300 s), 475 μL of "cold-chase solution" containing nonradioactive ATP and $MgSO_4$ was added (bringing the final concentrations to 5 mM ATP and 2.5 mM $MgSO_4$); then the solutions were quenched with 75 μL of 60% (w/v) $HClO_4$ 2 min later. Due to variation of the V_{max} of multisite catalysis at extreme pH values, this 2-min cold-chase period was adjusted to 1 min at pH 9.5 and 10 min at pH 5.5. "Acid-quench" experiments were similar (the same time points were taken, and F_1 and [γ-^{32}P]ATP concentrations were 2 and 0.2 μM, respectively), but the mixtures were directly quenched in 22 volumes of buffer containing 4.5 mM ATP, 2.3 mM $MgSO_4$, and 8.2% (w/v) $HClO_4$. [γ-^{32}P]ATP and [^{32}P]P_i were estimated as described by Grubmeyer et al. (1982) and Sugino and Miyoshi (1964).

(2) ATP Release (k_{-1}). The ATP release rate (k_{-1}) was assayed using a glucose–hexokinase trap as described by Duncan and Senior (1985) with a ratio of 2 μM F_1/0.2 μM ATP in the initial incubation mixture. The efficacy of trapping of ATP as glucose 6-phosphate was measured and found to

[1] Abbreviations: DTT, dithiothreitol; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; ΔG_{hyd}^{ATP}, Gibbs free energy of hydrolysis of ATP.

be very high in all the buffer systems used. This was expected from the pH insensitivity of hexokinase over the pH range 5.5–9.5 (Kaji & Colowick, 1965).

(3) P_i Release (k_{+3}) and the Equilibrium between Bound ATP and Bound P_i (K_2). These constants are determined by an "equilibration experiment" in which F_1 and [γ-^{32}P]ATP are mixed and bound ATP, bound P_i, and total ^{32}P are determined over a time scale up to 30–60 min. In previous work from this laboratory, this experiment was performed by mixing 1.5–3.0 mL of F_1 with an equal volume of ATP, with no special precautions taken to achieve rapid mixing, and with F_1/ATP concentration ratios of 1.0 or 0.3. While this technique would clearly *not* prevent an initial burst of cooperative catalysis (Penefsky, 1986), we reasoned this would not affect the results as long as a significant amount of ^{32}P became tightly bound to the F_1, such that the calculated values of K_2 and k_{+3} were dependable. Usually 20–30% of the [γ-^{32}P]ATP was bound [e.g., see Al-Shawi and Senior (1988) and Al-Shawi et al. (1989)]. Muneyuki et al. (1991) have recently questioned this assumption, preferring instead to conclude that *E. coli* F_1-ATPase shows heterogeneous hydrolysis of substoichiometric ATP, with the major fraction of ATP being hydrolyzed and products released rapidly, and only a small fraction (<20%) being hydrolyzed by the unisite pathway according to our previously published rate constants.

We carried out two types of experiment in order to determine what fraction of substoichiometric ATP is bound to *E. coli* F_1 when conditions to minimize cooperative catalysis are employed. First, using the pH 8.5 buffer described above, we carried out rapid mixing of equal (12.5 μL) volumes of F_1 and [γ-^{32}P]ATP exactly as in the "acid-quench/cold-chase" experiments with final F_1 and ATP concentrations of 2.0 and 0.2 μM (see section 1). The incubations were for 10, 20, and 40 s and 1, 5, 10, 15, 20, and 30 min. At the end of each incubation, 100 μL of buffer containing BSA (1.25 mg/mL) was added rapidly and mixed while vortexing for 2 s; then 100 μL of the mixture was applied to a centrifuge column, and the eluate was collected directly into 120 μL of 16% (w/v) $HClO_4$, 5 mM ATP, and 1 mM KH_2PO_4. Bound [γ-^{32}P]ATP and [^{32}P]P_i were estimated in the eluates as described above (Figure 1A of Results).

Second, the same experiment was repeated with 1 mM sodium azide present in both F_1 and [γ-^{32}P]ATP solutions before mixing (Figure 1B of Results).

As noted under Results, these two experiments demonstrated that when precautions are taken to minimize cooperative catalysis, most or all of the substoichiometric ATP is hydrolyzed by the standard unisite pathway. However, these assays are labor-intensive, and we desired a less time-consuming technique for routine use. The following procedure was therefore used to determine K_2 and k_{+3} at the various pH values.

A 1.5–2.0-mL volume of F_1 in the appropriate pH buffer (plus BSA, 2 mg/mL) was stirred extremely vigorously by magnetic stirring in a flat-bottomed vial. An equal volume of [γ-^{32}P]ATP was injected rapidly from a Gilson 5000P pipetman with wide-bore tip such that the final concentrations were 2 μM F_1 and 0.2 μM ATP. At appropriate time intervals (1 min up to 60 min), samples of 100 μL were taken, passed through centrifuge columns in the appropriate pH buffer supplemented with BSA (1 mg/mL), and collected directly into 120 μL of 16% (w/v) $HClO_4$, 5 mM ATP, and 1 mM P_i, and [^{32}P]P_i and [γ-^{32}P]ATP were estimated in the eluates as above. This method gave improved stoichiometry of bound ^{32}P, similar to that seen in Figure 1A, at all pH values tested. The equilibrium of bound P_i/ATP (K_2) was directly apparent, and the rate constant k_{+3} was calculated from the rate of decay of bound ^{32}P (Penefsky, 1986). Excellent first-order fits were obtained. The values for K_2 and k_{+3} obtained at pH 8.5 using this procedure were exactly the same as those obtained from Figure 1A.

(4) ADP Binding (k_{-4}) and Release (k_{+4}). The ADP binding rate (k_{-4}) was measured as described by Al-Shawi and Senior (1988). Briefly, [^{3}H]ADP and F_1 were incubated at 23 °C for varying times, binding was stopped by dilution, and bound [^{3}H]ADP was estimated by passing the samples immediately through centrifuge columns. F_1 concentrations used were 2–3 μM, and ADP concentrations were varied from 0.2 to 4.5 μM. k_{-4} was calculated from the initial linear phase of binding (~5 min) using a plot of ln ([free F_1]/[free ADP]). Since the $t_{1/2}$ for ADP release was of the order of 10 min, the initial linear phase slowed with longer time. The ADP release rate (k_{+4}) was measured as described by Al-Shawi and Senior (1988). Briefly, F_1 and [^{3}H]ADP were incubated together for 30 min, then the mixture was rapidly diluted in 40 volumes of stirred buffer containing 1 mg/mL BSA, and samples were passed through centrifuge columns to determine bound [^{3}H]ADP at time intervals ranging from 0 to 80 min. The F_1 and ADP concentrations in the initial incubation were 2–5 μM F_1 and 0.5–4.5 μM ADP. Preliminary tests showed that a nonradioactive ADP trap in the diluent buffer (0.3 μM) did not affect the results, and so it was omitted. k_{+4} was determined from first-order fits of decay of bound [^{3}H]ADP. It should be noted that these procedures yield K_d^{ADP} values which are in excellent agreement with K_d^{ADP} values determined by equilibrium dialysis or equilibrium binding/centrifuge column analysis, in which three or two equivalent exchangeable ADP binding sites were identified, respectively, in normal F_1 (Issartel et al., 1986; Al-Shawi et al., 1989).

(5) P_i Binding (k_{-3}). The P_i binding rate (k_{-3}) was *not* measured; rather, the $K_d^{P_i}$ was calculated from the equilibrium constants K_1, K_2, and K_4 and from ΔG_{hyd} for ATP hydrolysis under the conditions of the experiments; then k_{-3} was obtained from the measured k_{+3} and calculated $K_d^{P_i}$. ΔG_{hyd} values were from Rosing and Slater (1972) and Shikama and Nakamura (1973) (see Table III).

Multisite ATP Hydrolysis at Varied pH. F_1 was diluted 4-fold at least 1 h before use from storage buffer into assay buffer at the appropriate pH and 23 °C to give 4 μM concentration. Assays were initiated at 23 °C in a 0.5-mL volume by adding 5 μL of F_1 (final concentration in assay = 40 nM) and terminated with 0.5 mL of 10% (w/v) sodium dodecyl sulfate; then P_i was determined (Taussky & Shorr, 1953). The assay buffer was the same as the buffer for unisite catalysis (above) at pH 5.5, 6.5, 7.5, 8.5, and 9.5. For pH 5.0, 6.0, 7.0, 8.0, and 9.0, the unisite catalysis buffer that was 0.5 pH unit more alkaline was adjusted downward with H_2SO_4. Each of these buffers contained 0.5 mM $MgSO_4$. In order to clamp the free Mg^{2+} ion concentration at 0.5 mM in the presence of varied ATP concentration, supplemental $MgSO_4$ was added with ATP using the following ATP/Mg ratios: pH 7.0–9.5 inclusive, ratio (r) = 1.10; pH 6.5, r = 1.36; pH 6.0, r = 1.87; pH 5.5, r = 3.23; pH 5.0, r = 6.00. Total ATP concentration ranges used were as follows: pH 7.0–9.5 inclusive, 0.099–3.05 mM; pH 6.5 and 6.0, 0.198–6.10 mM; pH 5.5, 0.396–12.2 mM; pH 5.0, 0.78–23.3 mM. Total ATP was increased at low pH to sustain MgATP concentrations. Concentrations of ionic species of Mg, ATP, and MgATP were calculated as described by Fabiato and Fabiato (1979). It was confirmed that reactions were linear within the time scales used at each pH. At pH 5.0, nonlinearity was seen at times >25 min; at pH 9.5,

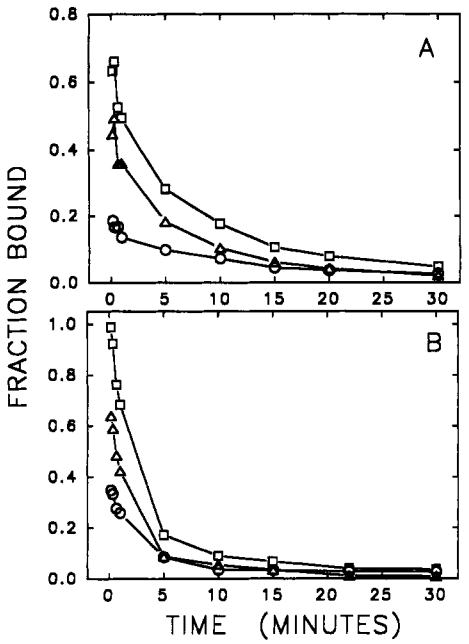

FIGURE 1: Hydrolysis of substoichiometric ATP under conditions designed to minimize cooperative catalysis. (A) Without added azide; (B) with 1 mM sodium azide. The experiment is described in detail under Materials and Methods. Briefly, equal (12.5 μL) volumes of F_1 and [γ-^{32}P]ATP in pH 8.5 buffer (±azide) were mixed rapidly. The final F_1/ATP concentration ratio was 2 μM/0.2 μM. At the times shown, 100 μL of pH 8.5 buffer plus 1.25 mg/mL BSA was added; 100-μL aliquots were taken and passed through centrifuge columns for analysis of bound [γ-^{32}P]ATP, bound [^{32}P]P_i, and total bound ^{32}P. (□) Total bound ^{32}P; (○) bound ATP; (△) bound P_i. Each point represents triplicate determinations. "Fraction bound" (*y* axis) refers to the fraction of the total (0.2 μM) [γ-^{32}P]ATP present.

nonlinearity was seen at times >2 min; thus measurements were made within these time limits. K_M and V_{max} values were obtained by fitting the kinetic data to the Michaelis–Menten equation by nonlinear least-squares regression analysis.

Radioactive ATP and ADP. [γ-^{32}P]ATP was obtained from Amersham and [2,8-^{3}H]ADP from NEN.

RESULTS

Hydrolysis of Substoichiometric ATP under Conditions Designed To Minimize Cooperative Catalysis. As described under Materials and Methods, Muneyuki et al. (1991) have recently criticized our previous work on unisite catalysis in *E. coli* F_1-ATPase, and it was necessary therefore to show that under the conditions used in the "acid-quench/cold-chase" experiments, most or all of the substoichiometric ATP was bound and hydrolyzed and products were released with rate constants characteristic of unisite catalysis.

Figure 1A shows an experiment in which equal (12.5 μL) volumes of F_1 and [γ-^{32}P]ATP were mixed rapidly to give a final concentration ratio of 2.0/0.2 μM at pH 8.5, and then the bound species were estimated by passage through centrifuge columns. Of the total ^{32}P, 66% was measurably bound at 20 s, and the calculated equilibrium constant for bound P_i/bound ATP is 2.5. Back-extrapolation of the total ^{32}P curve suggests that 66% is an underestimate of the true fraction of the ^{32}P bound to F_1 in the early seconds of the experiment. In a parallel "acid-quench" experiment performed under exactly the same conditions, 57% of the ATP was hydrolyzed at 20 s. Figure 1A shows that 49.4% of the ^{32}P was present as bound P_i at 20 s. Therefore, only a small fraction (7.6%) of the P_i had been released at 20 s. Bound P_i was released subsequently with a calculated dissociation rate (k_{+3}) of 3.5 × 10^{-3} s^{-1} and showed a very good fit to a first-order equation. These constants for K_2 and k_{+3} are very similar to those calculated from previous work, in which much lower stoichiometry of bound ^{32}P was attained, due to use of a different procedure (see Materials and Methods). The buffer used here did not contain P_i, which appears to account for the slight differences from previously reported constants (see Discussion). Our conclusion from this experiment was that when a manual rapid-mixing technique, small, equal volumes, and a high F_1/ATP concentration ratio (10/1) are used, cooperative catalysis on initial mixing is minimized such that most of the ATP is hydrolyzed by the unisite pathway as previously defined.

Figure 1B shows the same experiment as in Figure 1A, but with 1 mM sodium azide present. Sodium azide is an inhibitor of cooperative catalysis in *E. coli* F_1-ATPase, but it does not inhibit unisite catalysis (Noumi et al., 1987). Thus, we might hope to increase the fraction of bound ^{32}P, if an initial burst of cooperative catalysis was occurring. The bound ^{32}P increased to 99% in Figure 1B. The equilibrium constant (bound P_i/bound ATP) was 1.75. The rate of P_i release was 12 × 10^{-3} s^{-1} and showed a good first-order fit. Apparently, azide causes a 3.4-fold acceleration of this constant. Nevertheless, the experiment demonstrates that binding of ATP, equilibration of ATP and P_i on the enzyme, and slow release of products according to the unisite pathway are an intrinsic feature of all the molecules in our enzyme preparation.

Long-Term Stability of F_1 at pH 5.5–9.5. Since performance of the unisite assays could not be completed in less than 4 h after the initial equilibration of F_1 in unisite assay buffers, we estimated the long-term stability of F_1 in these buffers by adding 1.3–2.6 μL (2–4 μg) of enzyme (which had been previously equilibrated in unisite buffer and stood at 23 °C for 2 or 4 h) directly to 1 mL of ATPase assay buffer (50 mM Tris–sulfate, 5 mM $MgSO_4$, and 10 mM ATP at 30 °C and pH 8.5) and assaying P_i released at various times ranging from 30 s to 15 min. These assays showed that on standing in unisite buffer at pH 6.5, 7.5, and 8.5, no inactivation of F_1 occurred. At pH 9.5, inactivation of F_1-ATPase occurred (up to 25% after 2 h in pH 9.5 buffer and up to 50% after 4 h). This inactivation was fully reversible in the ATPase assay buffer described above with a $t_{1/2}$ of ~2 min as judged from curves of specific ATPase activity vs time. At pH 5.5, inactivation also occurred (up to 25% inactivation after 4 h in pH 5.5 buffer) which was also fully reversible ($t_{1/2}$ ~2 min) in assay buffer. The possible consequences of the observed inactivation on rate constant calculations are discussed below.

Possible Loss of Noncatalytic Nucleotides at Different pHs. The F_1 was purified and stored at −70 °C in buffer which initially contained 2 mM Mg^{2+} and 1 mM ATP, pH 7.0, so that the three noncatalytic nucleotide sites are expected to be filled (Perlin et al., 1984; Wise & Senior, 1985; Issartel et al., 1986). Before each unisite assay experiment, enzyme was preequilibrated in unisite buffer containing 0.5 mM Mg^{2+} with no added nucleotide. There was, therefore, the possibility that at different pHs, the noncatalytic sites might become "unloaded" during buffer equilibration and that subsequently, on addition of substoichiometric ATP or ADP during the unisite assays, all or some of this nucleotide may have entered noncatalytic sites. However, this appeared not to be the case. For example, on mixing of 0.2 μM [γ-^{32}P]ATP and 2 μM F_1, most of the ATP was rapidly bound, and virtually all of the bound radioactivity was "chased" by excess nonradioactive ATP in "cold-chase" experiments, showing that it resided in catalytic sites (Table I, columns 2 and 3). The exception was the experiment at pH 9.5. Since inactivation, probably due

Table I: Cold Chase of Bound [γ-^{32}P]ATP and Rapid Displacement of Bound [^{3}H]ADP by Excess MgATP[a]

	expt 1: % of bound ^{32}P committed to hydrolysis		expt 2: % displacement of bound [^{3}H]ADP	
pH	30 s	60 s	30 s	120 s
5.5	86.8	94.3	98.8	98.5
6.5	93.4	ND[b]	96.5	97.2
7.5	96.1	98.7	96.1	97.4
8.5	95.6	ND	95.3	97.1
9.5	54.4	62.5	79.1	84.6

[a] Experiment 1: Cold chase of [γ-^{32}P]ATP. 12.5-μL volumes each of F_1 and ATP were rapidly mixed as described under Materials and Methods (final concentrations = 2 μM F_1 and 0.2 μM ATP) and preincubated for 60 s (pH 7.5, 8.5, 9.5), 120 (pH 6.5), or 300 s (pH 5.5). Then (time zero) *either* 100 μL of buffer containing 1 mg/mL BSA *or* 100 μL of cold-chase solution (to bring the final concentrations to 5 mM ATP, 2.5 mM $MgSO_4$, and 1 mg/mL BSA) was added. 100-μL aliquots were passed through centrifuge columns at time zero (for buffer alone) or at times 30 or 60 s (for cold chase) and collected directly in 100 μL of 2 M HCl–1 mM KP_i. [γ-^{32}P]ATP and [^{32}P]P_i were estimated in the eluates. The percent of the bound ^{32}P that was displaced by 30 or 60 s of cold chase is shown in columns 2 and 3. Experiment 2: Bound [^{3}H]ADP displacement. In the [^{3}H]ADP displacement experiment, [^{3}H]ADP and F_1 were incubated as described under Materials and Methods to achieve binding and then passed through centrifuge columns to remove unbound ADP. 10-μL samples were mixed with 400 μL of buffer (pH 8.5) containing 5 mM ATP and 2.5 mM $MgSO_4$. After 30 or 120 s, samples (100 μL) were passed through centrifuge columns containing buffer plus 1 mg/mL bovine serum albumin to assure full recovery of F_1, and residual bound [^{3}H]-ADP was estimated. [b] Not determined.

to subunit dissociation (Wise et al., 1983), was shown to occur at pH 9.5, the lower "chase" at this pH is not unexpected. It may be due to [γ-^{32}P]ATP entering noncatalytic sites or entering catalytic sites which have become inactivated by the high pH. Also, as shown in Table I, columns 4 and 5, virtually all of the [^{3}H]ADP that was bound during ADP binding assays was "chased" rapidly by nonradioactive ATP, showing the ADP had entered catalytic sites. [The dissociation of ADP from noncatalytic sites has recently been shown to occur with $t_{1/2}$ = 1.35 h under similar buffer conditions at pH 8.0 (Pagan & Senior, 1990).] Again, the only exception was the pH 9.5 experiment, where apparently about 15–20% of the ADP entered into noncatalytic sites or into catalytic sites which had become inactivated. Further evidence in support of the view that entry of [γ-^{32}P]ATP or [^{3}H]ADP into noncatalytic sites was not significant was that in all of the ADP-release (k_{+4}) experiments, the release of [^{3}H]ADP could be fit very well by a monophasic curve; this was also true of the experiments in which [γ-^{32}P]ATP release (k_{-1}) was measured.

Overall, our conclusion was that loss of nucleotide from noncatalytic sites during preequilibration of enzyme with buffer of varied pH, causing binding of subsequently added [γ-^{32}P]ATP or [^{3}H]ADP to noncatalytic sites in unisite assays, was not a significant problem.

Rate Constants and Equilibrium Constants for Unisite Catalysis at Varying pH. All of the measured values, and the calculated k_{-3} and K_3 values, together with the assumed ΔG_{hyd} values for ATP hydrolysis, are shown in Tables II and III.

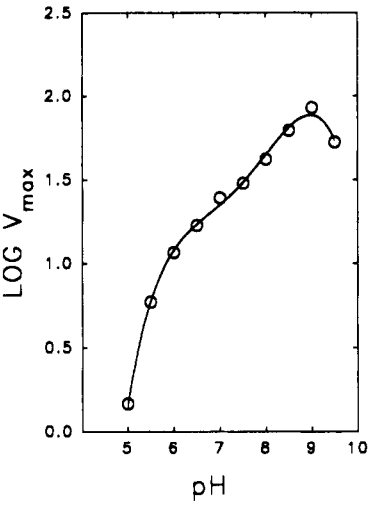

FIGURE 2: Multisite ATPase activity at varied pH. Multisite ATPase activity was measured in cationic acid buffers used for unisite catalysis assays with free [Mg^{2+}] clamped at 0.5 mM. The temperature was 23 °C (see Materials and Methods).

Calculations were performed to assess the affects of enzyme inactivation on the measured constants. First, it should be noted that none of the values at pH 6.5, 7.5, or 8.5 are subject to such error and that at pH 9.5 and 5.5, where some inactivation was seen, the rate constants subject to error would be k_{+1} and k_{-4}. Making the assumption that at pH 9.5, 50% of the F_1 was active, k_{+1} would increase by 2.1-fold and k_{-4} by 2.4-fold. At pH 9.5, for 75% of the F_1 being active, k_{+1} would increase by 1.35-fold and k_{-4} by 1.4-fold. At pH 5.5, for 75% of the F_1 being active, k_{+1} increases by 1.3-fold and k_{-4} by 1.2-fold. There would be corresponding changes in K_a^{ATP} (K_1) and K_d^{ADP} (K_4). Therefore, the errors introduced by the degree of inactivation of enzyme that was noted are small, and we did not correct the measured values for this factor.

Multisite ATP Hydrolysis by F_1-ATPase. The series of cationic acid buffers which was used to study unisite catalysis was also used to study multisite catalysis in the pH range 5.0–9.5 at 23 °C, with free [Mg^{2+}] clamped at 0.5 mM. Figure 2 shows a plot of log V_{max} vs pH obtained under these conditions. Between pH 5.5 and 9.0, multisite ATP hydrolysis accelerated by ~14-fold with a slope, $\Delta \log V_{max}/\Delta pH$, equal to 0.33. In Tris–sulfate or Tris–succinate buffers, as used in our earlier work to characterize the pH dependence of ATPase activity in normal and mutant *E. coli* F_1-ATPases at 30 °C [e.g., see Al-Shawi et al. (1988)], results were similar to those of Figure 2, with the slope, $\Delta \log V_{max}/\Delta pH$, equal to 0.5 (data not shown). A plot (not shown) of log (V_{max}/K_M) vs pH obtained in the cationic acid (unisite) buffers, using total ATP concentration for K_M calculation, showed the same inflexion points as in Figure 2 with the slope between pH 5.5 and 8.0 = 0.42. These data are qualitatively similar to those of Godinot and Penin (1981), who studied the pH dependence of

Table II: Rate Constants of Unisite Catalysis at Different pHs

pH	k_{+1} (M^{-1} s^{-1})	k_{-1} (s^{-1})	k_{+2} (s^{-1})	k_{-2} (s^{-1})	k_{+3} (s^{-1})	k_{-3} (M^{-1} s^{-1})	k_{+4} (s^{-1})	k_{-4} (M^{-1} s^{-1})
5.5	2.7×10^4	3.3×10^{-5}	1.3×10^{-1}	8.2×10^{-2}	9.1×10^{-4}	5.6×10^{-4}	9.4×10^{-4}	2.2×10^1
6.5	2.3×10^4	1.2×10^{-5}	8.2×10^{-2}	3.4×10^{-2}	4.5×10^{-4}	7.2×10^{-4}	2.1×10^{-3}	6.4×10^1
7.5	1.1×10^5	2.5×10^{-5}	1.2×10^{-1}	4.3×10^{-2}	1.2×10^{-3}	4.8×10^{-4}	1.6×10^{-3}	1.8×10^2
8.5	1.1×10^5	1.0×10^{-4}	1.8×10^{-1}	7.2×10^{-2}	3.7×10^{-3}	7.7×10^{-6}	1.2×10^{-3}	7.2×10^2
9.5	5.4×10^4	1.8×10^{-3}	1.4×10^{-1}	1.0×10^{-1}	5.6×10^{-3}	4.2×10^{-9}	3.7×10^{-4}	8.6×10^2
8.5 + P_i[a]	1.8×10^5	7.1×10^{-5}	5.6×10^{-2}	4.7×10^{-2}	1.0×10^{-3}	4.3×10^{-7}	3.4×10^{-4}	4.9×10^2

[a] Values obtained previously in 50 mM Tris–sulfate, 1 mM K_2HPO_4, and 0.5 mM $MgSO_4$, pH 8.5. Taken from Al-Shawi et al. (1989).

Table III: Equilibrium Constants of Unisite Catalysis and ΔG_{hyd}^{ATP} at Varying pHs

pH	K_a^{ATP} (K_1) (M^{-1})	K_2	K_d^{Pi} (K_3) (M)	K_d^{ADP} (K_4) (M)	$-\Delta G_{hyd}^{ATP}$ [a] (kJ/mol)
5.5	8.0×10^8	1.6	1.6	4.2×10^{-5}	28.1
6.5	1.9×10^9	2.4	0.62	3.3×10^{-5}	28.1
7.5	4.4×10^9	2.9	2.4	8.8×10^{-6}	30.7
8.5	1.1×10^9	2.5	4.8×10^2	1.7×10^{-6}	36.0
9.5	3.0×10^7	1.4	1.3×10^6	4.3×10^{-7}	41.7
8.5 + P_i [b]	2.6×10^9	1.2	2.3×10^3	6.9×10^{-7}	37.9 [b]

[a] Calculated from Figure 9 of Shikama and Nakamura (1973) and Table VIII and Figure 3 of Rosing and Slater (1972) for conditions I = 0.10, T = 25 °C, and [Mg] = 0.5 mM. [b] Values obtained previously in 50 mM Tris–sulfate, 1 mM K_2HPO_4, and 0.5 mM $MgSO_4$, pH 8.5. Taken from Al-Shawi et al. (1989). The previous value for ΔG_{hyd}^{ATP} is slightly higher than that used in this work.

mitochondrial F_1-ATPase in a similar series of buffers, with 1 mM Mg^{2+} present.

Discussion

Presence of Exogenous P_i Is Not Required for Unisite Catalysis in E. coli F_1 and Had Little Effect on Rates. From rows 4 and 6 of Tables II and III, one may compare the results obtained at pH 8.5 under conditions where P_i was absent (row 4) or present (row 6). The largest difference seen in any rate constant was ~3-fold (e.g., k_{+2}, k_{+3}, k_{+4}). Penefsky and Grubmeyer (1984), working with soluble bovine heart mitochondrial F_1, noted a strong activating effect of the oxyanions sulfite, chromate, and phosphate (but not sulfate) on hydrolysis of low concentrations of ATP and further showed that during unisite hydrolysis of ATP, the presence of P_i caused a large increase in the ATP association rate k_{+1}. Subsequently, however, Bullough et al. (1987), using a somewhat different preparation of bovine heart mitochondrial enzyme, saw little effect of P_i on k_{+1} and found that unisite catalysis proceeded without added P_i. Our experiments (Table II, column 2, row 4 vs row 6) showed a small effect of P_i on k_{+1}.

These considerations were of importance for this work, because at different pHs, different ratios of anionic P_i species might have had differing activating effects on unisite catalysis. However, the results showed clearly that P_i is not a required activator of unisite catalysis in *E. coli* F_1 and may be omitted from the reaction media.

Effects of pH on Steps of Unisite Catalysis. Figure 3 shows the pH dependence of step 1, the binding and release of ATP. The major change is in ATP dissociation (k_{-1}, Figure 3C), which accelerates at high pH, leading to a lowered affinity for ATP at pH 9.5 (Figure 3A). The highest affinity for ATP was at a physiological pH of 7.5 where $K_a^{ATP} = 4.4 \times 10^9$ M^{-1}. The apparent increase in k_{-1} at the higher pH values might be due to unfolding of the catalytic nucleotide-binding domain, or it might be due to a change in ionization (leading to increased net negativity) of a charged side chain within the catalytic site, which by extrapolation of the curves in Figure 3A,C would have a pK of ~8.0 (the slope of Δ log k_{-1}/ΔpH at higher pH values is around 1 in Figure 3C). As discussed below, there are grounds for favoring the latter conclusion. Notably, ATP association (k_{+1}) did not undergo a reciprocal deceleration at high pH.

Figure 4 shows parameters of step 2 as a function of pH. Step 2 is the catalytic step, and the findings show that the internal hydrolysis and resynthesis rates of bound ATP, and the overall equilibrium constant K_2, were essentially unaffected over the whole pH range tested. The catalytic unisite seems to be shielded from the medium, since over the pH range

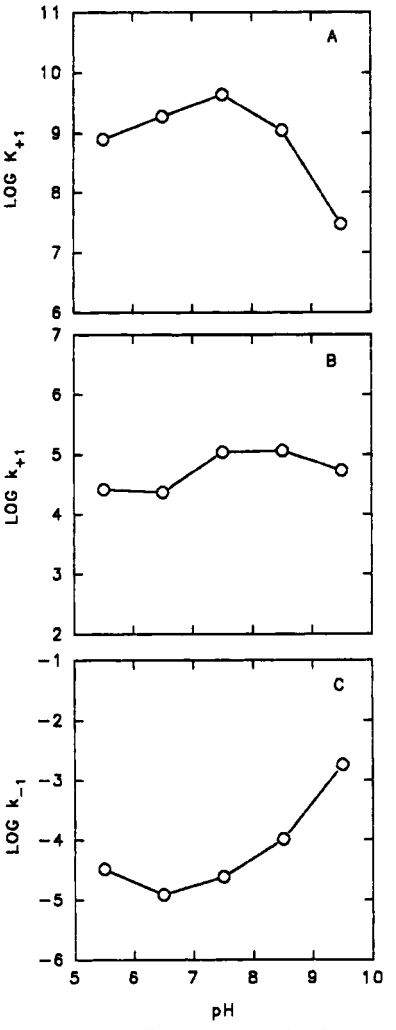

FIGURE 3: pH dependence of unisite ATP binding and release.

5.5–9.5 different anionic species of P_i, ADP, and ATP would be expected to occur, and to affect rate constants.

Figure 5 shows the pH dependence of parameters of step 3 (binding and release of P_i) during unisite catalysis. The effects on k_{+3} were small (Figure 5B). However, the rate constant k_{-3} (P_i binding) was markedly pH-dependent, being around 5×10^{-4} M^{-1} s^{-1} at pH 5.5–7.5, but then slowing by orders of magnitude at pH 8.5 and 9.5 (Figure 5C). As a consequence of changes in k_{-3} at high pH, K_d^{Pi} (K_3), which was around 1 M at pH 5.5–7.5, increased drastically at pH 8.5 and 9.5. We have previously suggested that binding of P_i (k_{-3}) into the catalytic site is a major energy-requiring step for ATP synthesis by ATP synthase, for the reason that this step is essentially forbidden in unisite catalysis (Al-Shawi & Senior, 1988; Al-Shawi et al., 1990). Our data for k_{-3} and K_3 (Figure 5) show that binding of P_i into the catalytic site in soluble *E. coli* F_1 is slow at any pH, is strongly pH-dependent with a transition point around pH 7.8, and requires higher concentrations of P_i than would normally be present in cells, supporting our argument that the P_i-binding step requires energy input from $\Delta\mu_{H^+}$. It appears from the k_{-3} rate values (Figure 5C) that $H_2PO_4^-$ is likely to be the actual species of P_i which is bound into the catalytic site during unisite ATP synthesis. Furthermore, at higher pH values, the slopes of Δ log K_3 and Δ log k_{-3} versus ΔpH are 2.1 and −2.0, respectively, suggesting there might be an ionizable enzyme group, with a pK_a around 8.4, which by generation of increased net negativity impairs P_i binding at high pH. It is noteworthy that a similar mechanism was implied to affect ATP disso-

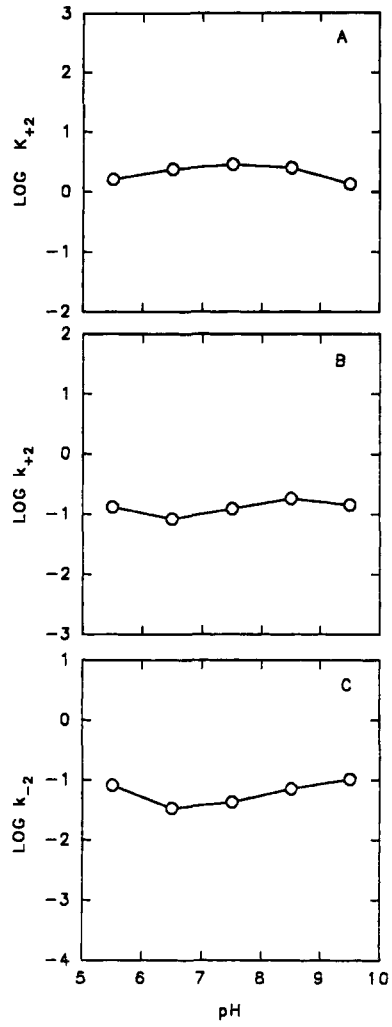

FIGURE 4: pH dependence of unisite catalytic interconversion of enzyme-bound ATP and ADP·P_i.

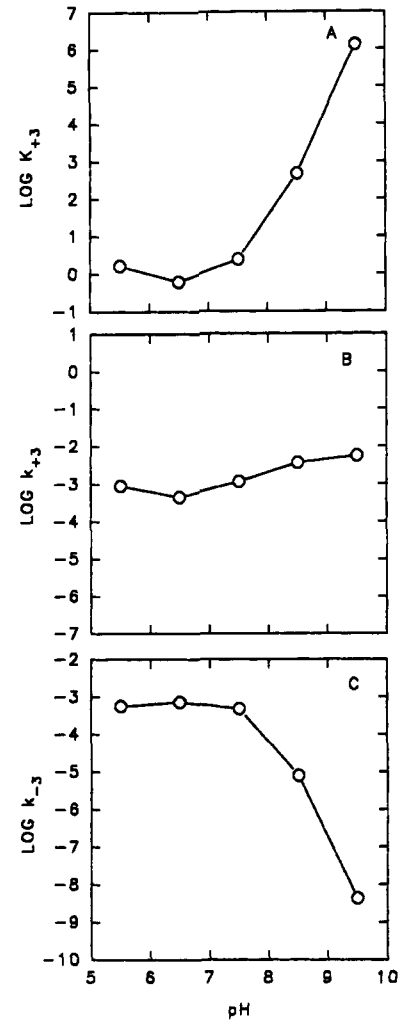

FIGURE 5: pH dependence of unisite P_i binding and release.

ciation (above) and also that P_i dissociation (k_{+3}) was not reciprocally accelerated at high pH.

Figure 6 shows effects of pH on unisite ADP binding and release. ADP release (k_{+4}) varied little over the range of pH tested (Figure 6B). Particularly, there was no large increase at higher pH values, in contrast with what was seen for ATP release (k_{-1}) (Figure 3C), suggesting that while oligomeric destabilization of F_1 did occur at high pH, unfolding of the catalytic site domain itself may not be the factor causing acceleration of k_{-1}. Rather, we presume that a group in the catalytic nucleotide-binding domain which normally interacts with ATP, but not with ADP, is responsible for the acceleration of k_{-1} at high pH. The ADP binding rate (k_{-4}) increased steadily over the whole pH range tested (Figure 6C), this result being in contrast to what was seen for ATP binding (k_{+1}) (Figure 3B). Again, this suggests that actual unfolding of the catalytic nucleotide-binding domain was not occurring in intact enzyme molecules at high pH. Further, the difference in pH dependence between k_{-4} (ADP binding) and k_{+1} (ATP binding), on the one hand, and also between k_{+4} (ADP release) and k_{-1} (ATP release) supports our previous suggestion that there are two different conformations of the catalytic site involved in unisite catalysis, one for ADP binding and one for ATP (and ADP·P_i) binding (Al-Shawi et al., 1990a). The data of Figure 6A demonstrate that K_d^{ADP} (K_4) is pH-dependent, ranging from 0.43 μM at pH 9.5 to 42 μM at pH 5.5. There was no strong dependence of ADP binding on the presence of P_i at pH 8.5 (K_d^{ADP} = 1.7 μM with no P_i and 0.69 μM with P_i; see Table III). Given that MgADP should be fully ionized at pH $\geq$7.5, we can conclude from Figure 6 that charged groups in the catalytic site promote ADP binding to the enzyme. The slope of Δ log k_{-4} vs ΔpH was 0.5.

Implications of the Results for the Mechanism of Catalysis in ATP Synthases. We have previously proposed (Al-Shawi et al., 1990a,b) that catalysis occurs in a sequestered, hydrophobic site and is driven by binding energy distributed over a catalytic site binding surface, implying the involvement of numerous side chains. We have suggested that as a result of specific stereochemical orientation and polarization of bound substrates, the reversible synthesis and hydrolysis of ATP may occur spontaneously. Data presented here show that between pH 5.5 and 9.5, no significant change in the unisite catalytic rate k_{+2} or k_{-2} occurs, supporting our proposal.

Multisite ATP hydrolysis in F_1-ATPase occurs at rates orders of magnitude larger than unisite ATP hydrolysis. The enhancement of the ATP $\rightarrow$ ADP + P_i cleavage reaction was up to $\sim$500-fold when the V_{max} of multisite hydrolysis is compared with k_{+2} in unisite catalysis (Figure 2 vs Table II), or up to $\sim 10^5$-fold when V_{max} is compared to unisite k_{+3} or k_{+4}. As was shown in Figure 2, multisite hydrolysis is significantly pH-dependent in *E. coli* F_1-ATPase, in contrast to unisite hydrolysis (Figures 3B, 4B, 5B, and 6B). Our data show, therefore, that the rate enhancements deriving from cooperative intersubunit interactions involve ionizable groups on the protein, and the slopes of plots of log (V_{max}/K_M) vs pH may imply that multiple such groups are involved. Given the known complexity of multisite catalysis in F_1-ATPases, it is

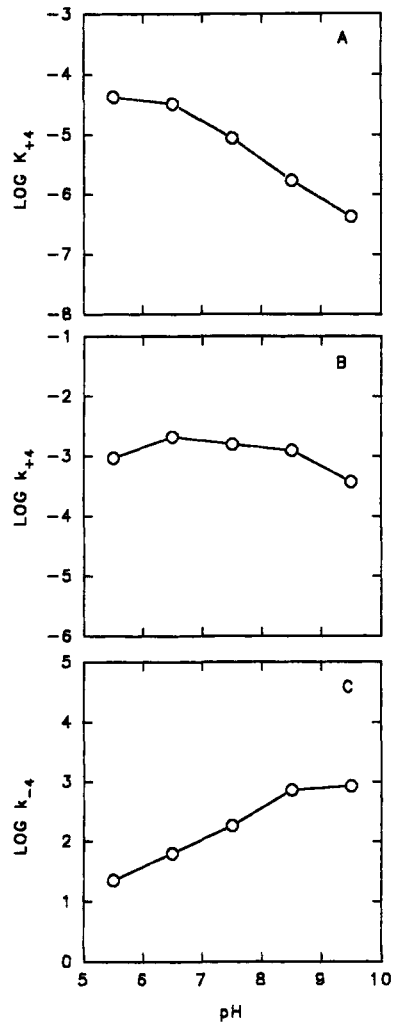

FIGURE 6: pH dependence of unisite ADP binding and release.

not possible to make direct pK_a assignments from our data. Further discussion of the possible inferences to be drawn from pH vs rate profiles for mitochondrial F_1-ATPase during multisite catalysis may be found in Godinot and Penin (1981) [see also Bullough et al. (1988)].

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

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