Defective Proton ATPase of *uncA* Mutants of *Escherichia coli*. 5′-Adenylyl Imidodiphosphate Binding and ATP Hydrolysis†

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ABSTRACT: The *Escherichia coli uncA* gene codes for the α-subunit of the F_1 sector of the membrane proton ATPase. In this work purified soluble F_1 enzymes from three mutant strains (*uncA401*, *uncA447*, and *uncA453*) have been compared to F_1 from a normal strain in respect to (a) binding of 5′-adenylyl imidodiphosphate (AMPPNP) to native enzyme in both the presence and absence of Mg, (b) high-affinity binding of MgATP to native enzyme, (c) total reloading of MgAMPPNP to nucleotide-depleted F_1 preparations, (d, e) ability to hydrolyze MgATP at both high MgATP concentrations (d) (steady-state conditions) and low MgATP concentrations (e) where substrate hydrolysis occurs under non-steady-state ("unisite") conditions, and (f) sensitivity of steady-state ATPase activities to inhibitors of normal F_1-ATPase activity. *uncA* mutant F_1 showed normal stoichiometry of MgAMPPNP binding to both native (three sites per F_1) and nucleotide-depleted preparations (six sites per F_1). Native *uncA* F_1 preparations showed lower-than-normal affinity for MgAMPPNP and MgATP at the first site filled. Binding of AMPPNP in the absence of Mg was similar to normal, except that no increase in affinity for AMPPNP was induced by aurovertin. The *uncA* F_1-ATPases had low but real steady-state rates of ATP hydrolysis, which were inhibited by aurovertin but relatively insensitive to inhibition by AMPPNP, efrapeptin, and sodium azide. Non-steady-state (unisite) ATP hydrolysis rates catalyzed at low substrate concentrations by *uncA* F_1-ATPases were similar to normal. The data strongly support the suggestion that $\alpha \leftrightarrow \beta$ intersubunit conformational interactions which are required for normal F_1-ATPase catalysis are severely attenuated in these *uncA* F_1 preparations.

The proton ATPase of *Escherichia coli* is a multisubunit membrane enzyme that functions as an ATP synthase during oxidative phosphorylation or as an ATP-driven transmembrane proton pump [see Downie et al. (1979) and Senior & Wise (1983) for reviews]. The enzyme consists of two sectors, an F_1 sector external to the membrane, on which ATP synthesis and hydrolysis occur, and a membrane sector responsible for proton transport and binding of F_1 to the membrane. The F_1 sector contains five different subunits designated α, β, γ, δ, and ε. Mutations in the *uncA* gene, which affect the α-subunit of F_1, lead to apparent loss of oxidative phosphorylation and ATPase activity (Downie et al., 1979).

A previous study of F_1 preparations from *uncA401*, *uncA447*, and *uncA453* mutant strains provided suggestive evidence that the catalytic derangement in the *uncA* F_1-ATPases was related to a lack of normal $\alpha \leftrightarrow \beta$ intersubunit conformational interaction (Wise et al., 1981). In that work intersubunit conformational interactions were followed by measuring the modulation of bound aurovertin fluorescence after the addition of a micromolar concentration of nucleotide (ADP) and by measuring the effect of bound aurovertin on affinity or nucleotide binding. The *uncA* F_1-ATPases were shown to be similar to normal F_1 in number and nature of adenine nucleotide binding sites (ADP and ATP) as studied and to have minimal alteration of chemical reactivity of essential residues in the β-subunits. The β-subunits have been suggested to contain all or part of the catalytic sites of F_1 (Senior & Wise, 1983).

Recent studies of mitochondrial F_1-ATPase preparations suggested that strong positive cooperativity between catalytic sites is responsible for the high rate of ATP hydrolysis normally observed when substrate is present at high concentration (millimolar range). Under these "multisite" conditions, up to three catalytic sites per F_1 were suggested to participate in catalysis (Cross et al., 1982; Gresser et al., 1982). When substrate concentrations were reduced to the micromolar range such that only the single high-affinity catalytic site (one per F_1-ATPase) bound MgATP ("unisite" conditions), very low rates of ATP hydrolysis were observed (Grubmeyer et al., 1982; Gresser et al., 1982). The enhancement of ATP hydrolysis between unisite and multisite conditions was estimated to be by 4–6 orders of magnitude (Cross et al., 1982; O'Neal & Boyer, 1983), suggesting that the cooperativity between catalytic sites in mitochondrial F_1 is indeed very strong.

One might therefore predict that the *uncA* F_1-ATPases would retain the ability to catalyze ATP hydrolysis at the very low unisite rate recently demonstrated in mitochondrial F_1 and may even retain residual elements of multisite ATP hydrolysis. We and others had previously assumed that loss of F_1-ATPase activity in the *uncA* mutants was total (Wise et al., 1981; Kanazawa & Futai, 1982). However, more recent work has uncovered an *uncA* F_1 preparation with 57% of normal ATPase activity,[1] showing that *uncA* mutants are not obligatorily *ATPase*⁻ (Wise, 1982).

In this study we present evidence that F_1 preparations from *uncA401*, *uncA447*, and *uncA453* mutant strains do exhibit very low ATP hydrolysis activity. Unisite ATP hydrolysis and attenuated multisite ATP hydrolysis in the *uncA* F_1-ATPases are examined, and the sensitivity of the multisite rates to known inhibitors of normal F_1-ATPase is presented. Additionally we have studied the catalytic site interactions further by measuring the binding of MgAMPPNP[2] (a substrate analogue) and MgATP to the native enzymes.

Materials and Methods

Materials. Sterox was obtained from Monsanto Chemicals; Malachite Green oxalate was obtained from C. Willis of the

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[1] From strains carrying the *uncA498* mutant allele.

[2] Abbreviations: AMPPNP, 5′-adenylyl imidodiphosphate; BSA, bovine serum albumen; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography.

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Biological Stain Commission, University of Rochester Medical Center; aurovertin was kindly donated by Dr. H. S. Penefsky and by Dr. R. B. Beechey; efrapeptin was a gift from Dr. R. Hamill, Eli Lilly Research Laboratories, Indianapolis. [γ-^{32}P]ATP was obtained from Amersham, and [^{3}H]AMPPNP and [α-^{32}P]AMPPNP were obtained from ICN. TLC plates were obtained from Brinkman Instruments. All other materials were obtained from commercial sources.

Growth of E. coli, Preparation of Cell Membranes, and Preparation of Purified Soluble F_1. Cells were grown to midexponential phase in 13-L batches in New Brunswick Microferm fermentors. All other methods were as described previously (Senior et al., 1979a,b; Wise et al., 1981).

Strains of E. coli Used. The *uncA* mutant strains AN718 (*uncA401*), AN1111 (*uncA447*), and AN1137 (*uncA453*) were described earlier (Senior et al., 1979a).[3] Normal (*unc*$^+$) F_1 was purified from strain AN1460 (Downie et al., 1980).

Determination of Reversion of uncA Mutant Alleles to unc$^+$ during Cell Growth. Serial dilution of samples of cell suspensions taken at the time of fermentor harvesting was performed; 0.1 mL of diluted samples was plated on supplemented minimal medium containing either succinate or glucose as carbon source. The number of colonies was determined after 3 and 7 days of incubation at 37 °C. Cell density at harvesting was between 3×10^9 and 9.5×10^9 cells/mL as judged from the glucose plates. Reversion frequencies to *suc*$^+$ phenotype (Cox & Downie, 1979) were calculated by dividing the number of colonies observed to grow on succinate by the number of cells plated.

ATP Hydrolysis Assays. ATPase assays were performed by colorimetry or by estimation of $^{32}P_i$ formed from [γ-^{32}P]ATP. Colorimetric assays (Lanzetta et al., 1979) were performed in 0.2 mL of 50 mM Tris–SO_4, pH 8.5, for 5–15 min. The reaction was stopped by addition of 0.8 mL containing Malachite Green oxalate (0.033% w/v), ammonium molybdate (1% w/v), HCl (1 N), and Sterox (0.04% w/v), followed exactly 1 min later by addition of 0.1 mL of sodium citrate (34% w/v). Absorbance (650 nm) was read at once. Under these conditions 2 nmol of P_i gave an absorbance of 0.12. Radioactive assays of ATPase activity were performed in 0.025 mL volumes in 1-mL microfuge tubes with additions as noted. Precipitation of P_i was performed by the method of Sugino & Miyoshi (1964) as modified by Grubmeyer & Penefsky (1981). The precipitate formed was washed, dissolved, and counted as described by Grubmeyer et al. (1982). Isotope trap-cold chase reactions were performed manually as described by Grubmeyer et al. (1982), except that ATP was 5 mM and $MgSO_4$ was 2.5 mM.

Preparation of Nucleotide-Depleted F_1. Removal of endogenous nucleotides from F_1 was performed as described by Garrett & Penefsky (1975) by gel filtration in 50% (v/v) glycerol buffer. F_1-containing column fractions were precipitated with 67% saturated $(NH_4)_2SO_4$, collected by centrifugation, and desalted before use by the centrifuge column technique (Penefsky, 1977). This enzyme retained full ATPase activity, and was added immediately to the nucleotide-containing binding incubation media.

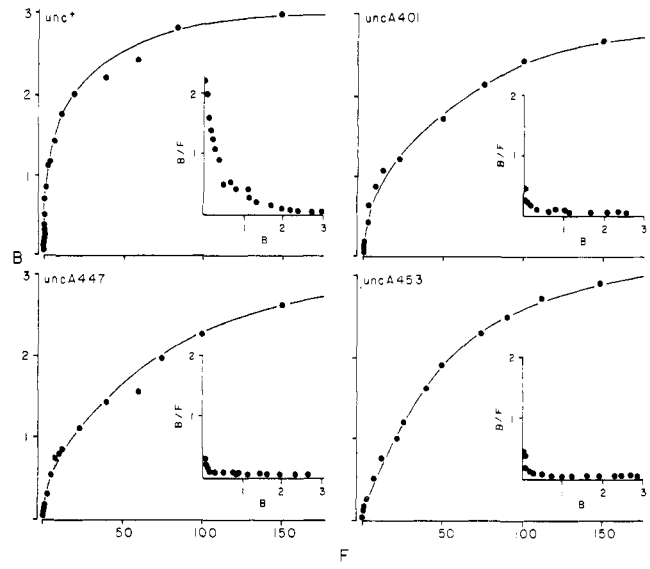

FIGURE 1: MgAMPPNP binding to native normal, *uncA401*, *uncA447*, and *uncA453* F_1 preparations. Shown are results of quadruplicate determinations of AMPPNP bound per F_1 (B, mol/mol) vs. free AMPPNP concentration (F, μM) with Scatchard analyses of the data (B/F vs. B) shown in the insets. Incubations were performed in 50 mM Tris–SO_4, 0.5 mM EDTA, and 2.5 mM $MgSO_4$, pH 7.5 at 23 °C.

Binding of Adenine Nucleotide Triphosphates to F_1 Preparations. AMPPNP binding experiments were performed as described by Wise et al. (1981) for ADP and ATP binding. Incubation periods were 2 h unless otherwise specified; 1 mg/mL BSA[2] carrier was included when AMPPNP concentrations were less than 5 μM (Cross & Nalin, 1982). Binding of [γ-^{32}P]ATP to F_1 was performed as described in the text by using the centrifuge column technique (Penefsky, 1977).

Estimation of F_1-Bound [γ-^{32}P]ATP and $^{32}P_i$. Quantitation of [γ-^{32}P]ATP and $^{32}P_i$ bound to F_1 after centrifuge column separation of incubation mixtures was performed either by specific precipitation of P_i (Sugino & Miyoshi, 1964) or by thin-layer chromatography. The two techniques gave identical results. Centrifuge column eluates were collected in test tubes containing 100 μL of 8% (w/v) $HClO_4$ or 2% (w/v) SDS, respectively. Thin-layer chromatography was performed on Polygram cel 300 PEI plates using 0.9 M LiCl–7 M urea as the mobile phase. Carrier nucleotides (ATP, ADP, and AMP) and carrier P_i, 35 nmol each, were added to samples before spotting. P_i was visualized by spraying developed plates with the ammonium molybdate solution of Taussky & Shorr (1953). Spots corresponding to each nucleotide, P_i, and the origin were scraped from the plates, incubated in 2 mL of 1 N Tris/1 N HCl for 1 h, and counted for radioactivity after addition of 10 mL of Amersham ACS. Average recovery of known amounts of [γ-^{32}P]ATP and $^{32}P_i$ from the plates was 84 and 82%, respectively.

Calculation of MgATP Concentrations. Calculations were performed by the computer program of Kohlbrenner & Cross (1979).

Results

5'-Adenylyl Imidodiphosphate Binding to Normal and uncA F_1 Preparations. Figure 1 shows AMPPNP binding data from experiments performed on "native" (i.e., not nucleo-

[3] Results of new genetic complementation tests communicated to us recently by L. Langman (Australian National University, Canberra, Australia) [performed as described by Gibson et al. (1977)] showed both AN1111 and AN1137 had the genotype *uncB*$^+$*E*$^+$*F*$^+$*H*$^+$*A*$^-$*G*$^+$*D*$^+$*C*$^+$. However AN718 had the apparent genotype *uncB*$^+$*E*$^+$*F*$^+$*H*$^-$*A*$^-$*G*$^+$*D*$^+$*C*$^+$, raising the possibility that both α- and δ-subunits of F_1 are abnormal in this strain. We prepared δ-depleted AN718 F_1 using the procedure of Smith & Sternweis (1977). When analyzed as described in Table III with 5.0 mM ATP/2.5 mM $MgSO_4$ as substrate, this preparation showed a P_i formation rate of 0.023 mol (mol of F_1)$^{-1}$ s^{-1}. δ-depleted *unc*$^+$ F_1 activity was 16 mol of P_i (mol of F_1)$^{-1}$ s^{-1} under the same conditions. Therefore, the catalytic impairment in AN718 is entirely referable to the α-subunit mutation. There is already conclusive evidence from in vitro complementation studies [reviewed in Dunn & Heppel (1981)] that *uncA401* α-subunit causes catalytic impairment of F_1.

tide-depleted) *unc*$^+$, *uncA401*, *uncA447*, and *uncA453* F_1 preparations in the presence of Mg. Approximately three sites per F_1 bound AMPPNP at the highest AMPPNP concentration in normal and in each of the *uncA* enzymes. Lower-than-normal affinity for AMPPNP at the first site filled is apparent for each of *uncA* F_1 enzymes, from both the "bound vs. free" and Scatchard data analyses. While strong apparent negative cooperativity between AMPPNP binding sites in *unc*$^+$ F_1 was observed (Figure 1), the apparent negative cooperativity between AMPPNP binding sites in the three *uncA* F_1 preparations seemed weaker. Hill equation coefficients suggested the presence of negative binding cooperativity in each of the F_1 preparations [for *unc*$^+$, $n = 0.69$; *uncA401*, 0.73; *uncA447*, 0.73; *uncA453*, 0.85; n equals the slope of log (bound/bound$_{max}$ − bound) vs. log (free AMPPNP)]. That the *uncA* F_1 preparations were similar to normal in native site stoichiometry, but different from normal F_1 in first site affinity for AMPPNP, suggests either that an alteration of binding site cooperativity in the *uncA* mutant F_1 enzymes has occurred or that an inherent structural asymmetry between the three native *unc*$^+$ sites has been modified in the *uncA* mutant enzymes.

We found only one site for binding of AMPPNP to normal, native F_1 in the absence of added Mg, with a K_d of about 160 μM (data not shown). Thus, the effect of omission of Mg was to increase the K_d and reduce n to 1. This is reminiscent of our previous work showing reduction in n and increase of K_d for ADP binding to *unc*$^+$ F_1 when Mg was absent (Wise et al., 1981). Aurovertin (10 μM) modulated the binding of AMPPNP in the absence of added Mg by decreasing K_d to 101 μM without appreciably affecting n. This is similar to the effect of aurovertin to increase the affinity for ADP or ATP by *unc*$^+$ F_1 which we found previously (Wise et al., 1981).

We did not construct binding curves for AMPPNP binding to the native *uncA* F_1 preparations in the absence of added Mg, but the amount bound at selected concentrations of AMPPNP (data not shown) confirmed that the *uncA* mutant F_1 enzymes were similar to normal in this property. Aurovertin had no effect on AMPPNP binding to *uncA* F_1 enzymes in contrast to its effect on normal F_1. This again was consistent with the lack of effect of aurovertin on ADP or ATP binding to *uncA* F_1 which we noted previously (Wise et al., 1981).

In AMPPNP binding experiments performed on nucleotide-depleted normal, *uncA401*, *uncA447*, and *uncA453* F_1 preparations, incubation with 2 mM AMPPNP resulted in 6.0, 5.8, 5.9, and 5.7 AMPPNP bound per respective F_1 (mol/mol) (quadruplicate experiments). Saturation was achieved within 1 h, and no increase above six AMPPNP per F_1 was observed in incubations as long as 2.5 h.

In summary, the results of the AMPPNP binding studies confirmed that the *uncA401*, *uncA447*, and *uncA453* F_1-ATPases have a normal stoichiometry of adenine nucleotide binding sites and showed an abnormally low affinity for MgAMPPNP at the first native nucleotide site in each of the *uncA* F_1 enzymes.

F_1-Bound Substrate and Product after Incubation of Normal or uncA F_1 with [γ-^{32}P]ATP at Equimolar Concentration. An investigation of [γ-^{32}P]ATP binding to the F_1-ATPases was conducted under conditions that would elucidate substrate binding to high affinity native sites. Figure 2 (upper panel) shows the amount of ^{32}P bound per F_1 (mol/mol) when 0.3 μM F_1 and 0.3 μM [γ-^{32}P]ATP were incubated together for the indicated times. With *unc*$^+$ F_1 we observed an increase in the amount of F_1-bound ^{32}P initially which reached a maximal level at approximately 1 min and was followed by a slow decay over the remainder of the incubation period.

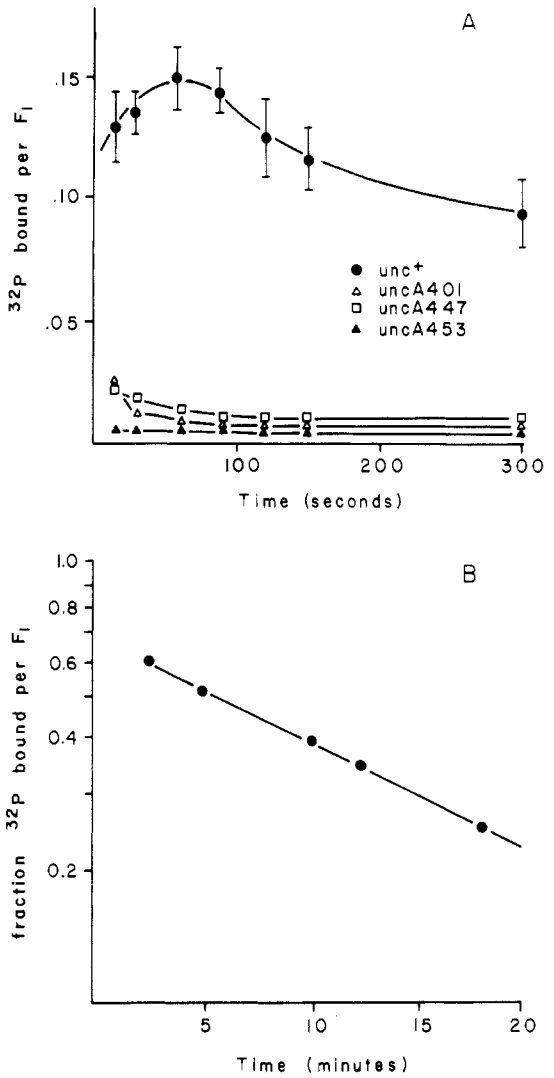

FIGURE 2: High-affinity ATP binding to normal, *uncA401*, *uncA447*, and *uncA453* F_1 preparations. (Panel A) F_1 (0.3 μM) and [γ-^{32}P]ATP (0.3 μM) were incubated in 50 mM Tris–SO_4, 1 mM KP_i, and 0.5 mM $MgSO_4$, pH 8.5, for the indicated times at 23 °C; the incubations were then passed through centrifuge columns equilibrated with the same buffer (Penefsky, 1977). Results presented (mol of ^{32}P/mol of F_1) are averages of quadruplicate determinations, except *unc*$^+$ experiments which are averages of six determinations (error bars represent calculated standard deviations). The *unc*$^+$ data have been corrected for a slow linear loading of nonexchangeable sites observed for normal F_1 over the incubation time course (0.06 mol/mol of F_1 at 5 min) by subtracting the amount of nonexchangeable ^{32}P bound per F_1 that is not committed to hydrolysis (Grubmeyer et al., 1982). No nonexchangeable binding was observed with the three *uncA* F_1-ATPases. (Panel B) F_1 (*unc*$^+$ only) and [γ-^{32}P]ATP were incubated under the conditions described for panel A for 2.5 min after which centrifuge columns were used to separate bound from free ligand. Eluates were aged for the indicated times at 23 °C; free ligand in the aged eluates was removed by reapplication of the centrifuge column technique. Results shown are averages of quadruplicate determinations. The value 1.0 corresponded to 0.12 ^{32}P bound per F_1 (mol/mol).

Figure 2 (upper panel) also shows parallel incubations of *uncA401*, *uncA447*, and *uncA453* F_1 preparations. Very low amounts of F_1-bound ^{32}P were observed for each of the *uncA* enzymes. Since normal rates of $^{32}P_i$ formation by *uncA* enzymes were observed under similar incubation conditions (see below), the data of Figure 2 suggest that ^{32}P dissociation rates are abnormally high in the *uncA401*, *uncA447*, and *uncA453* F_1-ATPases. This is consistent with the earlier demonstration of lower-than-normal affinity for the ATP analogue, AMPPNP, at the first native nucleotide binding site in the *uncA* F_1-ATPases.

Table I: Specific ATPase Activity of *uncA* Mutant F_1 Preparations[a]

F_1 preparation		ATPase (nmol min^{-1} mg^{-1})[b]	inhibition by aurovertin (%)[c]	enzyme concn in assay (μg/mL)
uncA401:	prep 1	14.0	75	173
	prep 2	14.5	56	100
uncA447:	prep 1	95	25	54
	prep 2	120	18.5	60
uncA453:	prep 1	84	85	59
	prep 2	127	75	41
unc$^+$		64 000	88	0.25
		61 600	ND[d]	1.1
		20 800	76	12
		20 200	ND	25

[a] All assays were carried out at least 6 times each. Average values are shown. The rate of phosphate released was linear throughout the assays for each of the enzymes tested. [b] Enzyme was preincubated at 30 °C for 3 min in 50 mM Tris-SO_4, pH 8.5, then ATP and $MgCl_2$ were added to final concentrations of 5 and 2.5 mM, respectively, and incubation was continued 5–15 min. P_i liberated was determined colorimetrically as described under Materials and Methods. [c] Enzyme was preincubated as above with aurovertin (5 μM) added as an ethanol solution. Controls contained ethanol. Final ethanol concentration was kept <1% (v/v) since it was inhibitory toward *uncA447* and *uncA453* F_1 at higher concentrations. ATP and $MgCl_2$ concentrations were 2.5 and 1.25 mM, respectively. [d] ND, not determined.

In separate experiments performed on *unc*$^+$ F_1 under the same incubation conditions, ^{32}P dissociation after removal of unbound ligand by centrifuge column treatment occurred at a dissociation rate of 1.5×10^{-3} s^{-1} (Figure 2, lower panel). This result indicates that P_i dissociation from a single site on *E. coli unc*$^+$ F_1 occurs at a similar rate to that shown for mitochondrial F_1 (Grubmeyer et al., 1982). No effect on ^{32}P dissociation was observed when hexokinase (0.58 mg/mL) and glucose (50 mM) were included in the centrifuge column eluates that were aged, suggesting that [γ-^{32}P]ATP dissociation from and rebinding to F_1 contributed little to the observed ^{32}P dissociation kinetics. Analysis of F_1-bound [γ-^{32}P]ATP and $^{32}P_i$ after centrifuge column treatment of *unc*$^+$ incubations (same conditions) either by specific precipitation of P_i (Sugino & Miyoshi, 1964) or by direct TLC analysis of the centrifuge column eluates showed that the fraction of F_1-bound ^{32}P present as [γ-^{32}]ATP was 0.60 ± 0.09 (SD) and 0.61 ± 0.06 (SD) after 1 and 2.5 min of incubation (five experiments each). These values are very similar to the values reported for F_1-bound substrate–product equilibrium in mitochondrial and chloroplast F_1 (Grubmeyer et al., 1982; Feldman & Sigman, 1982), confirming that normal *E. coli* F_1 behaves very similarly to normal F_1-ATPases from other sources in these assays.

ATP Hydrolysis Activities of F_1 Preparations from uncA401, uncA447, and uncA453 Mutant Strains. Table I shows the ATPase activities (under multisite assay conditions) of three *uncA* F_1 preparations and compares them to normal (*unc*$^+$) F_1. The specific ATPase activity of normal F_1 depends on the concentration of F_1 in the assay, due to dissociation of the ε-subunit at low F_1 concentrations with accompanying activation of ATP hydrolysis (Sternweis & Smith, 1980; Dunn & Heppel, 1981). Table I shows, in agreement with the previous reports, that the specific ATPase activity of normal F_1 was maximal at 0.25 μg/mL and decreased over the range 1–10 μg/mL. Increasing the enzyme concentration above 10 μg/mL did not further affect specific activity. The *uncA* F_1 preparations were each assayed at enzyme concentrations greater than 10 μg/mL. Average specific ATPase activities of 0.07% (*uncA401*), 0.5% (*uncA447*), and 0.5% (*uncA453*) of normal (*unc*$^+$) specific activity (at 12–25 μg/mL) were observed (Table I).

The *uncA401* and *uncA453* activities were inhibited by aurovertin (5 μM) to nearly the same extent as normal (*unc*$^+$) F_1, while the *uncA447* ATPase activity was less sensitive to aurovertin than normal F_1 (Table I). Satre et al. (1978, 1980) showed that aurovertin is a specific inhibitor of F_1-ATPase activity in *E. coli*. The fact that aurovertin inhibited the ATPase activities of the *uncA401* and *uncA453* F_1 preparations therefore suggests strongly that the low ATPase activity observed for the *uncA401* and *uncA453* preparations was intrinsic to the F_1-ATPase. The *uncA447* F_1 preparations were more abnormal in that ATP hydrolysis was less sensitive to aurovertin inhibition than normal F_1. Further experiments were conducted to check for the presence of non-F_1 contaminant ATP-hydrolyzing enzymes. Hydrolysis of *p*-nitrophenyl phosphate (5 mM) and AMP (5 mM) by the *uncA* F_1 preparations was tested by using each substrate under the same conditions used for assaying hydrolysis of ATP in Table I. There was no detectable hydrolysis of either *p*-nitrophenyl phosphate or AMP by any of the *uncA* F_1 preparations. These experiments suggest that the ATP hydrolysis activities observed were not due to contaminating phosphatase or nucleotidase enzymes in the F_1 preparations. Furthermore, none of the activities in Table I was inhibited by 1 mM sodium vanadate.

It was also necessary to demonstrate that the low ATPase activities of the *uncA401*, *uncA447*, and *uncA453* F_1 preparations were not attributable to normal F_1 present as a result of spontaneous reversions during cell growth. At the time of harvesting, samples of the cell suspensions were analyzed for the frequency of reversion to *suc*$^+$ phenotype. Reversion frequencies for the three *uncA* alleles were as follows: *uncA401*, 1 in 5×10^6; *uncA447*, 1 in 7×10^4; *uncA453*, 1 in 3×10^4. From these values, assuming a specific activity for *unc*$^+$ F_1 of 20 μmol min^{-1} mg^{-1}, it was calculated that the maximal ATPase activity due to (reverted) *unc*$^+$ F_1 in the *uncA* F_1 preparations would be the following: *uncA401*, 0.004 nmol min^{-1} mg^{-1}; *uncA447*, 0.3 nmol min^{-1} mg^{-1}; *uncA453*, 0.7 nmol min^{-1} mg^{-1}. These values represent 0.03%, 0.3%, and 0.6% of the actual ATPase activity of the respective *uncA* F_1 preparations shown in Table I.

In summary, the low ATP hydrolysis activities reported in Table I for the *uncA401* and *uncA453* F_1 preparations seem to be attributable to the *uncA* F_1-ATPases themselves. The evidence is less complete in the case of the *uncA447* enzyme, which was relatively insensitive to inhibition by aurovertin. It seems worth noting, however, that it might be considered unusual if a contaminant with ATPase activity copurified only with the *uncA447* F_1 preparations, and not with the other two *uncA* F_1 preparations, since the three *uncA* mutant strains are isogenic with respect to all except the *unc* genes.

Abnormal Sensitivity of uncA F_1 Preparations to Inhibitors of Normal F_1-ATPase Activity. Results of experiments are shown in Table II. AMPPNP is a potent competitive inhibitor of normal *E. coli* ATPase activity [K_i = 0.6 μM at pH 7.5 (Wise et al., 1983)] and inhibited normal F_1 strongly at 10 μM concentration. The three *uncA* F_1-ATPase activities were only weakly inhibited at 10 μM AMPPNP, and the degree of inhibition did not change when the ATP concentration was reduced to as low as 50 μM. Efrapeptin is a relatively weak inhibitor of normal *E. coli* F_1 as compared to mitochondrial F_1 (Wise et al., 1983). As seen in Table II, at 84 μM efrapeptin, normal F_1-ATPase was inhibited by 76%. In contrast the *uncA* ATPase activities were only weakly inhibited in parallel experiments. Sodium azide is a potent inhibitor of

Table II: Sensitivity of *uncA* Mutant F_1 Preparations to Inhibitors of Normal *Escherichia coli* F_1-ATPase Activity[a]

F_1 preparation	inhibition (%) 1 mM NaN_3	10 μM AMPPNP[b]	84 μM efrapeptin
AN718 (*uncA401*)	<2	12.0	10.5
AN1111 (*uncA447*)	<2	12.5	18.0
AN1137 (*uncA453*)	<2	6.2	6.0
AN1460 (*unc*$^+$)	97.5	89	76

[a] Assays were carried out as in Table I. F_1, which had been preequilibrated with 50 mM Tris-SO_4, pH 7.5, was preincubated in 50 mM Tris-SO_4, pH 8.5, with inhibitor for 3 min at 30 °C. The reaction was started by addition of ATP (to 2.5 mM) and $MgCl_2$ (to 1.25 mM). The concentration of enzyme was 12–15 μg/mL (*unc*$^+$ F_1) or as described in Table I (*uncA* F_1). Efrapeptin inhibition assays were performed in plastic test tubes. All values are averages of at least quadruplicate estimations. [b] When Mg^{2+} was included together with AMPPNP during preincubation, the reaction was started by addition of ATP, and similar results were obtained.

Table III: Comparison of P_i Formation Rates of Normal and *uncA* F_1-ATPases[a] at ATP Concentrations of 5 mM and 0.3 μM

F_1	P_i formation rate[b] (ATP/Mg concn) (0.3 μM/ 0.5 mM)	(5.0 mM/ 0.5 mM)	(5.0 mM/ 2.5 mM)
unc$^+$	0.025	45.5 (1820×)	47.6 (1900×)
uncA401	0.025	0.022 (0.9×)	0.034 (1.4×)
uncA447	0.025	0.29 (12×)	0.27 (11×)
uncA453	0.027	0.088 (3×)	0.14 (5×)

[a] Incubation of 1 μM F_1 with [γ-^{32}P]ATP in 50 mM Tris-SO_4/ 1 mM KP_i, pH 8.5, with $MgSO_4$ and ATP concentrations indicated was performed at 23 °C. P_i formed was estimated as described under Materials and Methods for radioactive assay of ATPase activity, and P_i formation rates were calculated. Rates of 0.3 μM ATP were estimated from the initial velocity tangents shown in Figure 4. Rates at 5 mM ATP (with 0.5 or 2.5 mM $MgSO_4$) were estimated from linear time courses of P_i formation for each of the F_1-ATPases. Total product formed was less than 10% of initial substrate in each case. Numbers in parentheses show the P_i formation rate enhancement (equals rate at 5 mM ATP divided by rate at 0.3 μM ATP). [b] Moles of P_i per mole of F_1 per second.

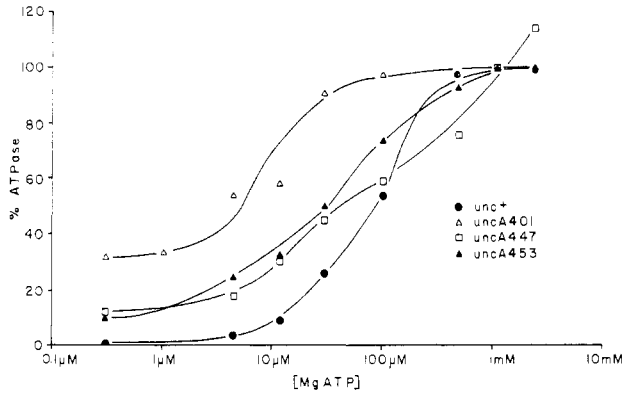

FIGURE 3: Relative rates of ATP hydrolysis by normal, *uncA401*, *uncA447*, and *uncA453* F_1 preparations. F_1 preparations (preequilibrated by the centrifuge column technique with 50 mM Tris-SO_4, pH 7.5) were incubated at a final concentration of 0.05 μM for 3 min at 30 °C in 50 mM Tris-SO_4, pH 8.5. ATPase reaction was started by addition of ATP or [γ-^{32}P]ATP and $MgCl_2$ in a 2/1 molar ratio. P_i liberated was measured by colorimetry (above 30 μM MgATP) or by precipitation of $^{32}P_i$ (below 30 μM MgATP). Control experiments at 30 μM MgATP gave the same results with the two techniques. Results presented are averages of duplicate determinations. *uncA447* F_1 was not saturated at 2.5 mM MgATP but did saturate at 5 mM MgATP (data not shown).

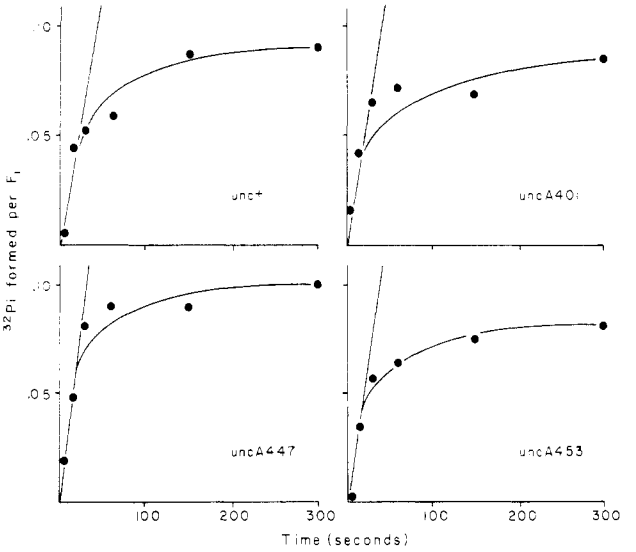

FIGURE 4: P_i formation by normal, *uncA401*, *uncA447*, and *uncA453* F_1 preparations. F_1 (1.0 μM) and [γ-^{32}P]ATP (0.3 μM) were incubated in 50 mM Tris-SO_4, 1.0 mM KP_i, and 0.5 mM $MgSO_4$, pH 8.5 at 23 °C, for the indicated times. The reaction was stopped by addition of 22 volumes of buffer containing 5 mM ATP, 2.5 mM $MgSO_4$, and 8% (w/v) perchloric acid. $^{32}P_i$ formed was estimated by precipitation of P_i (Sugino & Miyoshi, 1964). Results shown ($^{32}P_i$ formed per F_1, mol/mol) are averages of quadruplicate determinations. The *closed circles* represent actual experimental measurements. The *curves* are calculated logarithmic regression lines fitted to the data. The *straight lines* are tangents to the calculated regression curves.

normal F_1. Thus, it was remarkable that none of the *uncA* F_1-ATPase activities were inhibited by 1 mM NaN_3.

Rate of ATPase Activity as a Function of MgATP Concentration. In Figure 3 we have shown the ATPase activity of each enzyme at MgATP concentrations ranging from 0.29 μM to 2.5 mM at an enzyme concentration of 0.05 μM. The activity at 1.25 mM MgATP was set equal to 100% for each enzyme.

It is important to note here that this 100% value was orders of magnitude higher for the *unc*$^+$ enzyme than for the *uncA* enzymes when expressed in absolute rates (Table I and Table III). Figure 3 shows that as the MgATP concentration was increased, the relative ATPase increased in a sigmoidal fashion for each of the *uncA* enzymes as well as for the *unc*$^+$ enzyme. Recent investigations of the mechanism of ATP hydrolysis in mitochondrial F_1 discuss the influence of strong positive catalytic cooperativity between three catalytic sites (Cross et al., 1982; Gresser et al., 1982), and the sigmoidal curves seen in Figure 3 are consistent with such cooperativity. The results shown suggest that residual catalytic cooperativity is retained in the *uncA* preparations, although it is much reduced in extent, and further that the *uncA* preparations differ one from another in this respect.[4]

Non-Steady-State ATP Hydrolysis by Normal, uncA401, uncA447, and uncA453 F_1 Preparations at Low Substrate Concentrations. Figure 4 shows formation of $^{32}P_i$ by the F_1-ATPases when 1 μM F_1 was incubated with 0.3 μM [γ-

[4] It is interesting to note that a partial revertant strain AN718RR2 (obtained from parent strain AN718, *uncA401*) yielded an F_1 preparation with specific ATPase activity (5 mM ATP/2.5 mM $MgSO_4$) of 230 nmol min^{-1} mg^{-1}. When the rate of ATPase was plotted against MgATP concentration, AN718RR2 F_1 behaved unlike either *unc*$^+$ F_1 or the parent *uncA401* F_1. Rather it behaved similarly to *uncA447* F_1 in Figure 3. Genetic complementation studies (L. Langman, personal communication) showed strain AN718RR2 is defective in α-subunit of F_1, but all other subunits are normal.

^{32}P]ATP. The figure shows that P_i formation by the *uncA* F_1-ATPases was very similar to normal. Very similar rates were observed when the ATP concentration was 1 μM. Control experiments containing no F_1 indicated that less than 1% of available [γ-^{32}P]ATP was nonezymatically hydrolyzed over the 5-min incubation period.

Comparison of Steady-State (Multisite) and Non-Steady-State (Unisite) ATP Hydrolysis by Normal and uncA F_1 Preparations. Table III compares the P_i formation rates determined for *unc*$^+$, *uncA401*, *uncA447*, and *uncA453* F_1-ATPases at ATP concentrations of 0.3 μM and 5 mM and an F_1 concentration of 1 μM. Very similar-to-normal initial ATP hydrolysis rates were observed for the *uncA* enzymes when the ATP concentration was 0.3 μM. However, the *uncA401*, *uncA447*, and *uncA453* enzymes showed much lower-than-normal enhancement of ATP hydrolysis rates when the substrate concentration was raised to 5 mM. The *uncA* F_1-ATPase enzymes therefore appear to be defective in the catalytic cooperativity effect. It should be noted that the P_i formation rate enhancement observed here for normal *E. coli* F_1 on shifting from unisite to multisite conditions is approximately 1–2 orders of magnitude less than the P_i dissociation rate enhancements reported for mitochondrial F_1 (Cross et al., 1982; O'Neal & Boyer, 1983). This is because in our assays we are measuring both F_1-bound and released P_i under unisite conditions.

Discussion

We have previously suggested (Wise et al., 1981) that MgADP, ADP, and ATP (no Mg) binding sites in F_1-ATPase enzymes prepared from strains carrying the *uncA401*, *uncA447*, and *uncA453* mutant alleles were similar in number and nature to those of normal *E. coli* F_1. The work presented here showed that each of the *uncA* ATPase enzymes, like the normal F_1, had a total of six binding sites for MgAMPPNP, of which three were available to bind in the native (i.e., not nucleotide-depleted) enzyme. It appeared that the binding of MgAMPPNP at the first site in native normal F_1 occurred with higher affinity than at the other two sites and that in the *uncA* F_1-ATPases the binding of the first MgAMPPNP occurred with lessened affinity as compared to normal enzyme (Figure 1). It was then shown that whereas normal F_1 bound [γ-^{32}P]ATP with high affinity at the first binding site in the presence of Mg, the *uncA* F_1-ATPases had lower-than-normal binding of substrate at this site (Figure 2).

Cross & Nalin (1982) showed that native mitochondrial F_1 binds MgAMPPNP at three sites, each of which appears to be a catalytic site. One of these sites has much higher affinity than the others, and the affinity of this site may be modulated by binding of ADP at a separate catalytic site (Nalin & Cross, 1982). Very high affinity substrate (MgATP) binding to the first site in native mitochondrial F_1 has been studied in detail recently (Grubmeyer et al., 1982; Cross et al., 1982) and has been suggested to be an important feature of catalysis by the F_1-ATPase.

Thus, it would seem that the lowered affinity of the first substrate binding site in the *uncA* F_1-ATPases is intimately related to the catalytic derangement. However, this need not necessarily involve an inherent structural change of a unique high-affinity site; the data are also consistent with the idea that conformational interactions between catalytic sites which give apparent negative binding cooperativity in normal F_1 are abnormal in the *uncA* F_1-ATPase enzymes.

The results of measurements of AMPPNP binding to native, normal F_1 in the absence of Mg confirmed what we had found previously with ADP (Wise et al., 1981), i.e., that Mg has a profound effect on the affinity of *E. coli* F_1 for adenine nucleotides. In fact, in the absence of Mg, only one AMPPNP binding site with $K_d \sim 160$ μM was seen in normal F_1. The *uncA* F_1 preparations appeared similar to normal in this respect. The lack of any effect of aurovertin on binding of AMPPNP in the *uncA* F_1 preparations, in contrast to its effect on normal F_1, confirms what we had already suggested (Wise et al., 1981; Senior & Wise, 1983) that an intersubunit conformational interaction between an aurovertin site and a nucleotide binding site is interrupted in the *uncA* mutant F_1. In our earlier work (Wise et al., 1981) we had also studied this conformational interaction in the reverse direction (i.e., ADP-induced enhancement of bound aurovertin fluorescence) and had shown that this was an *inter*subunit event and not an *intra*subunit event.

Recent studies of mitochondrial F_1-ATPase have suggested that strong positive catalytic cooperativity between the active sites enhances very low unisite ATPase rates to high multisite hydrolysis rates when more than one catalytic site is occupied by substrate (see introduction). In studies presented here we have shown that normal, *uncA401*, *uncA447*, and *uncA453* F_1-ATPases hydrolyze ATP at similar very low rates when the substrate concentration is low and available to only one catalytic site per F_1 (Figure 4). When the concentration of ATP was raised such that normal F_1 demonstrated high steady-state ATPase activity, little enhancement of ATP hydrolysis rates over unisite rates was observed for the *uncA401*, *uncA447*, and *uncA453* F_1-ATPases (Tables I and III). These results strongly suggest that the mutations in the *uncA* F_1-ATPases attenuate normal positive catalytic cooperativity and that defective cooperativity between catalytic sites in the *uncA401*, *uncA447*, and *uncA453* F_1-ATPases is responsible for the low levels of observed catalysis in these enzymes. The sigmoidal relationship between relative ATPase activity and MgATP concentration (Figure 3) suggests that limited cooperativity between catalytic sites does occur in the *uncA* F_1-ATPases.

The observed insensitivity of the *uncA* F_1-ATPase activities to inhibition by sodium azide and the much reduced inhibition by AMPPNP and efrapeptin (Table II) suggest that the mechanism of inhibition by these agents may involve perturbations of normal catalytic cooperativity. That the three mutant *uncA* preparations were only slightly inhibited by AMPPNP and efrapeptin (both of which are thought to bind at a catalytic site) could also be consistent with the observations presented that indicate altered affinity for substrate and substrate analogue at the presumptive first catalytic site of the three *uncA* F_1-ATPases.

In summary, we feel the new data presented here strongly support our earlier hypothesis (Wise et al., 1981) that the $\alpha \leftrightarrow \beta$ intersubunit conformational interaction is defective in these *uncA* F_1-ATPase enzymes and that it is the attenuation of this conformational interaction which is responsible for lessened catalytic rates, as discussed in more detail elsewhere (Senior & Wise, 1983). The three *uncA* mutant alleles *uncA401*, *uncA447*, and *uncA453* presumably affect residues essential for cooperative interactions between catalytic sites, possibly at interfacial regions, and from the detailed properties described here, they appear to be different mutations.

Acknowledgments

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Registry No. ATPase, 9000-83-3; ATP, 56-65-5; MgATP, 1476-84-2; AMPPNP, 25612-73-1; MgAMPPNP, 69977-25-9; au-

rovertin, 11002-90-7; efrapeptin, 56645-91-1; sodium azide, 26628-22-8.

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Interactions of Cholesterol Hemisuccinate with Phospholipids and $(Ca^{2+}$-$Mg^{2+})$-ATPase†

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ABSTRACT: Cholesterol hemisuccinate has been shown to equilibrate readily with liposomes and with the $(Ca^{2+}$-$Mg^{2+})$-ATPase from sarcoplasmic reticulum and has been used to modify the sterol content of these membranes. Cholesterol hemisuccinate incorporates into dioleoylphosphatidylcholine (DOPC) up to a molar ratio of 3:1 sterol to DOPC. Effects on lipid order as detected by electron spin resonance and fluorescence polarization are comparable to those of cholesterol. Binding constants have been determined, and the uncharged form of the sterol binds more strongly than the anionic form. Binding to DOPC and to the lipid component of the ATPase system is comparable. From use of the fluorescence quenching properties of 1,2-bis(9,10-dibromooleoyl)phosphatidylcholine and dibromocholesterol hemisuccinate, two classes of binding sites on the ATPase have been deduced. At the lipid/protein interface, the binding constant for cholesterol hemisuccinate is considerably less than that for DOPC. At the second set of sites (nonannular sites), binding occurs with K_d = 0.55 in molar ratio units. The effect of cholesterol hemisuccinate on the activity of the ATPase depends on the phospholipid present in the system: ATPase reconstituted with DOPC is inhibited whereas ATPase reconstituted with dimyristoleoylphosphatidylcholine is activated. We conclude that changes in membrane fluidity are not important in determining ATPase activity in these systems.

Membrane fluidity has been thought to be an important determining factor in a variety of membrane processes (Shinitzky & Henkart, 1979; Kates & Kuksis, 1980). It is known that many organisms alter the phospholipid compositions of their membranes in response to a variety of environmental changes, and it has been suggested that these changes occur in order to maintain an optimal fluidity for the membrane (Sinensky, 1974, 1980). A classic example is provided by the increase in saturation of the phospholipid acyl chains in bacteria with increasing growth temperature (McElhaney, 1982; Melchior, 1982). Cholesterol is also postulated to be

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