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Properties of F_1-ATPase from the *uncD412* mutant of *Escherichia coli*

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Properties of purified F_1-ATPase from *Escherichia coli* mutant strain AN484 (*uncD412*) have been studied in an attempt to understand why the amino acid substitution in the β-subunit of this enzyme causes a tenfold reduction from normal MgATP hydrolysis rate. In most properties that were studied, *uncD412* F_1-ATPase resembled normal *E. coli* F_1-ATPase. Both enzymes were found to contain a total of six adenine-nucleotide-binding sites, of which three were found to be non-exchangeable and three were exchangeable (catalytic) sites. Binding of the non-hydrolysable substrate analogue adenosine 5'-[$\beta\gamma$-imido]triphosphate (p[NH]ppA) to the three exchangeable sites showed apparent negative co-operativity. The binding affinities for p[NH]ppA, and also ADP, at the exchangeable sites were similar in the two enzymes. Both enzymes were inhibited by efrapeptin, aurovertin and p[NH]ppA, and were inactivated by dicyclohexylcarbodi-imide, 4-chloro-7-nitrobenzofurazan and *p*-fluorosulphonylbenzoyl-5'-adenosine. K_m values for CaATP and MgATP were similar in the two enzymes. *uncD412* F_1-ATPase was abnormally unstable at high pH, and dissociated into subunits readily with consequent loss of activity. The reason for the impairment of catalysis in *uncD412* F_1-ATPase cannot be stated with certainty from these studies. However we discuss the possibility that the mutation interrupts subunit interaction, thereby causing a partial impairment in the site–site co-operativity which is required for 'promotion' of catalysis in this enzyme.

The membrane proton-ATPase (H^+-transporting ATPase) of *Escherichia coli* is a large multisubunit enzyme which catalyses ATP synthesis in oxidative phosphorylation and ATP-driven generation of a transmembrane proton gradient. The F_1-sector of the proton-ATPase is the part of the enzyme carrying the catalytic sites for ATP synthesis and hydrolysis, and it is constructed of five different types of subunit, designated α–ε. The membrane sector of the enzyme (also called 'F_0') is responsible for transport of protons across the membrane, and contains three different types of subunit. All eight subunit polypeptides are encoded by genes in the *unc* operon (reviewed by Gibson, 1983; Senior & Wise, 1983).

Fayle *et al.* (1978) showed that the β-subunit of F_1-ATPase is encoded by the *uncD* gene. Subsequently it has become apparent that strains of *E. coli* carrying mutations in the *uncD* gene are of considerable potential importance, because the β-subunits carry all or part of the catalytic sites for ATP hydrolysis and synthesis in the F_1-sector (reviewed by Senior & Wise, 1983). Mutations in the *uncD* gene have been shown to cause a wide range of phenotypic effects, including interruption of assembly of the F_1-sector of the enzyme (Fayle *et al.*, 1978; Senior *et al.*, 1979*b*; Cox *et al.*, 1981), blockage of proton conduction caused by unusually tight binding of the abnormal β-subunits to the membrane-sector subunits (Fayle *et al.*, 1978; Senior *et al.*, 1979*b*), enhanced affinity of binding between the F_1-sector and the membrane sector (Senior *et al.*, 1983), and synthesis of an F_1-sector of normal molecular size and subunit composition, but with impaired catalytic activity (Senior *et al.*, 1979*b*; Kanazawa *et al.*, 1980; Senior *et al.*, 1983). Mutations in the *uncD* gene giving this last effect are likely to be of value in studying the mechanism of ATP synthesis and hydrolysis for the reason that they presumably cause alterations at single, functionally important amino acid residues in the

Abbreviations used: DCCD, dicyclohexylcarbodi-imide; Nbf-Cl, 4-chloro-7-nitrobenzofurazan; p[NH]ppA, adenosine 5'-[$\beta\gamma$-imido]triphosphate; SDS, sodium dodecyl sulphate; FSBA, *p*-fluorosulphonylbenzoyl-5'-adenosine; DMSO, dimethyl sulphoxide.

F_1-ATPase β-subunit, and a functionally-impaired F_1-ATPase may be prepared in soluble homogeneous form for study.

The mutant *uncD412* allele directs the synthesis of an F_1-sector of normal size and subunit composition, but with approximately one-tenth of normal MgATPase activity. The β-subunit of the *uncD412* F_1 was shown to have an altered isoelectric point, but no further characterization was performed (Senior *et al.*, 1979*b*). In this paper we present a detailed study of the *uncD412* F_1-ATPase and discuss the possible nature of the functional derangement.

Methods

Preparation of soluble F_1-ATPase

Soluble *uncD412* F_1 was purified from *E. coli* strain AN484 as described previously (Senior *et al.*, 1979*a*,*b*), except that, in the ion-exchange-chromatography and gel-filtration steps, 2mM-$MgCl_2$ was substituted for 2mM-EDTA in the buffers. Although in the previous study, *uncD412* F_1-ATPase was not seen to dissociate (Senior *et al.*, 1979*b*), here a tendency of the enzyme to depolymerize during the gel-filtration step of the purification procedure was noted, with depolymerized F_1 subunits eluting separate from, and later than, the F_1-ATPase peak. However, the addition of $MgCl_2$ to the buffers, as suggested by Kanazawa *et al.* (1980), overcame this problem. The ATPase activity of the purified *uncD412* F_1 remained unchanged after storage for 3 months at −20°C in 50mM-Tris/HCl, 10% (v/v) glycerol, 1mM-dithiothreitol, 40mM-6-aminohexanoic acid, 40mM-Na_2SO_4, 2mM-$MgCl_2$, 1mM-ATP, pH 7.4. Normal F_1-ATPase was purified from strain AN862 as described previously (Senior *et al.*, 1979*a*, *b*).

Assay of ATPase activity

ATPase activity was measured at pH 7.5 and at 30°C, in 50mM-Tris/H_2SO_4/5mM ATP/2.5mM-$MgCl_2$ (modifications to this assay are described where pertinent in Tables and Figure legends). Approx. 5μg of enzyme was added to 0.5ml of assay medium to initiate the reaction, which proceeded for 5min (normal F_1-ATPase) or 30min (*uncD412* F_1-ATPase) and was stopped by addition of 0.5ml of 10% (w/v) SDS. P_i liberated was estimated by the procedure of Taussky & Shorr (1953).

Aurovertin fluorescence measurements

The techniques for measurement of bound aurovertin fluorescence were as described in Wise *et al.* (1981).

Reaction of F_1-ATPase with the covalent inhibitors DCCD, Nbf-Cl and FSBA

The techniques for reaction of F_1-ATPase with radioactive DCCD and Nbf-Cl were as described previously (Wise *et al.*, 1981). For reaction with FSBA, F_1-ATPase (0.5mg/ml) was first equilibrated in 10% (v/v) DMSO/50mM-Tris/succinate, pH 7.0, by the centrifuge column technique (Penefsky, 1977). The enzyme was stable for at least 3h in this buffer at 30°C, and FSBA concentrations up to 3mM could be added without precipitation. After FSBA addition, ATPase activity was assayed at intervals to determine rate of inactivation. [^{3}H]FSBA was used to label normal and *uncD412* F_1-ATPases. The reaction conditions were as described above, but at the end of the reaction a large excess of β-mercaptoethanol was added to quench the FSBA that had not reacted. The protein was precipitated by the addition of 3 vol. of saturated $(NH_4)_2SO_4$, cooled on ice to 0°C, and centrifuged. The pellets were redissolved in buffer containing 6M-guanidium chloride, 0.1M-dithiothreitol, 40mM-Tris/H_2SO_4, pH 7.5, and 1mM-EDTA, and passed through columns of Sephadex G-25 (0.9cm × 25cm) in buffer containing 6M-urea, 0.1M-dithiothreitol, 40mM-Tris/H_2SO_4, pH 7.5, and 1mM-EDTA. The protein peak was collected and dialysed for 24h against the urea-containing buffer (200vol.) with changes at 2h and 5h before determination of bound radioactive FSBA.

Preparation of nucleotide-depleted F_1-ATPase

The procedure of Garrett & Penefsky (1975), involving passage of F_1-ATPase through Sephadex columns in buffer containing 50% (v/v) glycerol, was followed in principle in order to prepare nucleotide-depleted enzyme. F_1-ATPase samples [2–5mg in 0.1ml of 50% (v/v) glycerol/100mM-Tris/H_2SO_4/4mM-EDTA, pH 8.0] were applied to a column (0.9cm × 60cm) of Sephadex G-25 equilibrated with the same buffer and eluted at 1.8ml/h at room temperature. Peak fractions (A_{280}/A_{260} ratio > 1.80) were pooled, precipitated with 67%-satd. $(NH_4)_2SO_4$ and collected by centrifugation. The protein was dissolved in 0.1ml of 50mM-Tris/H_2SO_4/0.5mM-EDTA, pH 8.0, and desalted before use on a centrifuge column in the same buffer at room temperature.

Reloading of nucleotide-depleted F_1 samples with p[NH]ppA

The procedure followed in principle that described by Cross & Nalin (1982). Nucleotide-depleted samples of F_1-ATPase (0.25–0.5mg/ml) were incubated in 50mM-Tris/H_2SO_4/0.5mM-EDTA/2.5mM-$MgSO_4$, pH 8.0, with 2mM-[^{3}H]p[NH]ppA at room temperature. At deter-

mined times (see the Results section), enzyme and bound nucleotide were separated from free nucleotide by passage through centrifuge columns equilibrated with 50mM-Tris/H_2SO_4/0.5mM-EDTA/2.5mM-$MgSO_4$, pH8.0. Protein content and radioactivity of the eluates were measured to allow calculation of total amount of p[NH]ppA bound (in mol) per mol of F_1-ATPase.

Exchange of p[NH]ppA out of nucleotide-reloaded F_1-ATPase samples by addition of ATP or ADP

The procedure followed in principle that of Cross & Nalin (1982). To samples of F_1-ATPase that had been maximally reloaded with p[NH]ppA as described above, non-radioactive ATP (1mM) or ADP (10mM) was added and incubation was carried out at room temperature for various times. Bound p[NH]ppA was separated from free p[NH]ppA by centrifuge column-elution as described above, and the final centrifuge-column eluate was assayed for radioactivity and protein content. The nucleotide remaining bound corresponds to non-exchangeable ('tightly bound') nucleotide, and exchangeable nucleotide corresponds to that displaced by non-radioactive ATP or ADP in the second incubation.

Binding of ADP, ATP and p[NH]ppA to native F_1-ATPase preparations

Radioactive ADP, ATP or p[NH]ppA were incubated with native (ie. not nucleotide-depleted) F_1-ATPase, and binding was measured as described by Wise *et al.* (1981). Bovine serum albumin (1mg/ml) was included as carrier to prevent loss of F_1-ATPase on the centrifuge columns (Cross & Nalin, 1982).

Materials

[2,8-^{3}H]ADP and [2,8-^{3}H]ATP were obtained from New England Nuclear. [U-^{14}C]Nbf-Cl and [*carbodi-imide*-^{14}C]DCCD were obtained from Research Products International. Aurovertin D was a gift from Dr. H. Penefsky, Public Health Research Institute, New York City, NY, U.S.A., Dr. P. Linnett and Dr. R. B. Beechey, both of Shell Research Laboratories, Sittingbourne, Kent, U.K. Efrapeptin was a gift from Dr. R. Cross and Dr. R. L. Hamill (Lilly Research). FSBA (non-radioactive) was obtained from Sigma. [2,8-^{3}H]FSBA was synthesized from [2,8-^{3}H]adenosine (New England Nuclear) and *p*-fluorosulphonylbenzoyl chloride (Aldrich) as described by Wyatt & Colman (1977).

Routine procedures

Assays of protein concentration and other routine procedures were as described previously (Wise *et al.*, 1981). A molecular weight of *E. coli* F_1-ATPase of 382000 was assumed in calculations (Senior & Wise, 1983).

Results

Catalytic properties of uncD412 F_1-ATPase

The K_m values derived from linear Lineweaver-Burk plots were similar for both enzymes hydrolysing either MgATP or CaATP (Table 1). The *uncD412* F_1-ATPase had a $V_{max.,\,CaATP}/V_{max.,\,MgATP}$ ratio of 2.55, whereas this ratio was equal to 0.17 in normal F_1-ATPase. This property was examined further by changing the Ca^{2+} or Mg^{2+} concentration while keeping ATP concentration constant, as shown in Fig. 1. This graph shows a clear difference between the mutant and normal F_1-ATPase. p[NH]ppA inhibited *uncD412* F_1-ATPase and normal *E. coli* F_1-ATPase competitively, with linear Lineweaver-Burk plots intercepting at the *y*-axes. As Table 1 shows, the $K_{i,p[NH]ppA}$ was the same in both enzymes. Efrapeptin showed 'mixed linear inhibition' (Segel, 1976) of both enzymes, with linear Lineweaver–Burk plots intercepting in the upper-left quadrant. The K_i and αK_i (Segel, 1976) values calculated are shown in Table 1. Efrapeptin is clearly much less inhibitory toward *E. coli* F_1-ATPase than towards ox heart mitochondrial F_1-ATPase. Cross & Kohlbrenner (1978) cite a K_d of 10^{-8}M for binding of efrapeptin to the latter enzyme. Aurovertin D was an uncompetitive inhibitor of both *uncD412* and normal F_1-ATPase (Lineweaver–Burk plots were linear and parallel), with similar inhibitory potency toward each enzyme. When the percentage inhibition of ATP hydrolysis

Table 1. *Comparison of catalytic properties of uncD412 and normal F_1-ATPase*

In 1–4 the assay conditions were as described in the Methods section, except that [ATP] was varied from 0.1 to 2.5mM, the ATP/metal-ion ratio being kept at 2:1. $CaCl_2$ was substituted for $MgCl_2$ as required. In 5, conditions were as in 1–4, with Mg^{2+} as cation. p[NH]ppA was varied from 0.1 to 10μM. K_i was calculated from a slope replot of linear Lineweaver–Burk plots (Cleland, 1970). In 6 and 7 the assay conditions were as in 1–4. Plastic tubes were used for all assays. [Efrapeptin] was varied from 1 to 50μM. K_i and αK_i were calculated as described by Segel (1976).

	uncD412 F_1-ATPase	Normal F_1-ATPase
1. $K_{m,CaATP}$ (mM)	0.67	0.48
2. $K_{m,MgATP}$ (mM)	0.22	0.37
3. $V_{max.,CaATP}$ (μmol/min per mg)	2.01	1.17
4. $V_{max.,MgATP}$ (μmol/min per mg)	0.79	6.8
5. $K_{i,p[NH]ppA}$ (μM)	0.59	0.60
6. $K_{i,efrapeptin}$ (μM)	13.2	21.5
7. $\alpha K_{i,efrapeptin}$ (μM)	69	44

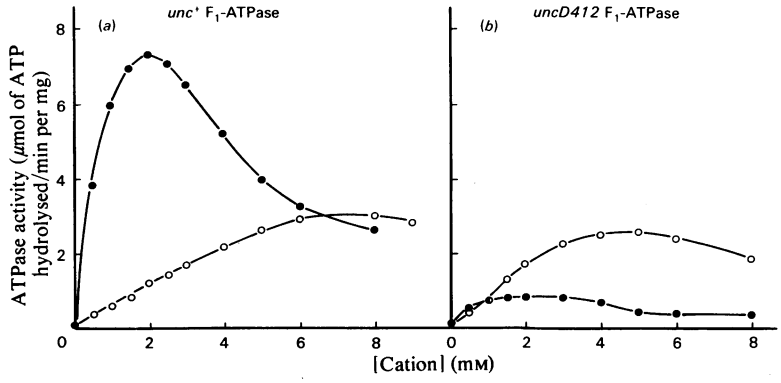

Fig. 1. *Comparison of CaATP and MgATP as substrates for uncD412 and normal F_1-ATPase*
ATPase activity was measured in 50mM-Tris/H_2SO_4/5mM-ATP, pH7.5; the cation concentration (●, Mg^{2+}; ○, Ca^{2+}) was varied as shown.

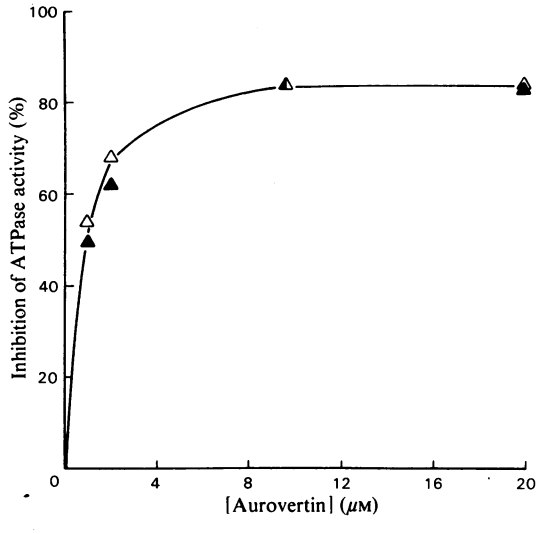

Fig. 2. *Aurovertin D inhibition of (△) uncD412 and (▲) normal F_1-ATPase*
ATPase activity was measured in 50mM-Tris/H_2SO_4/5mM-ATP/2.5mM-$MgCl_2$, pH7.5, the aurovertin concentration being varied as indicated. Aurovertin was added as an ethanolic solution and controls contained the same amounts of ethanol as experimental tubes.

was plotted against increasing aurovertin concentration at saturating ATP concentration, the curves obtained with both normal and *uncD412* F_1-ATPase coincided. An inhibition of 50% was seen at about 1μM-aurovertin D (Fig. 2), in agreement with previous studies (Satre *et al.*, 1980). We conclude that the aurovertin-binding site is normal in *uncD412* F_1ATPase.

The rate of ATP hydrolysis at different pH values between 7.3 and 9.3 was measured. Normal *E. coli* F_1-ATPase showed a sigmoidal increase in hydrolysis rate as the pH increased from 7.3 to 9.3, with an inflexion point at 8.3 (see Senior *et al.*, 1983). The *uncD412* F_1-ATPase showed a bell-shaped curve of activity against pH, with a maximum at pH8.3 (results not shown). There was therefore a marked difference in behaviour between the two enzymes. In order to test whether *uncD412* F_1-ATPase dissociated at high pH, gel-filtration experiments were carried out. Samples of normal and *uncD412* F_1-ATPase (0.27mg in 0.1ml) were passed through a column (0.9cm × 60cm) of Sephacryl S-300 equilibrated in 50mM-Tris/H_2SO_4/2.5mM-$MgCl_2$/5mM-ATP (ie. same as ATPase assay medium) at pH7.2 and pH9.3. The elution rate was 6ml/h at room temperature. Fractions were assayed for ATPase activity and protein content. Normal F_1-ATPase eluted as a single symmetrical peak of both protein and ATPase activity at both pH7.2 and pH 9.3. *uncD412* F_1-ATPase at pH7.2 showed slight dissociation into subunits, with 95% of the protein being eluted coincidentally with ATPase activity and 5% of the protein being eluted in later fractions. The *uncD412* F_1-ATPase at pH9.3 showed extensive dissociation into subunits, with <40% of the protein being eluted coincidentally with ATPase activity and >60% of the protein being eluted in later fractions. We conclude, therefore, that the *uncD412* mutation confers a pH-sensitive instability on the F_1-ATPase, and at high pH causes depolymerization and consequent loss of activity.

Inactivation and labelling of essential residues of uncD412 F_1-ATPase by DCCD and Nbf-Cl

DCCD and Nbf-Cl were found to inactivate *uncD412* F_1-ATPase to the same extent as normal F_1-ATPase, and the β-subunit was labelled pre-

Table 2. *Inactivation and labelling of uncD412 and normal F_1-ATPases by DCCD and Nbf-Cl*
The reaction conditions were as follows: Expt. A: [^{14}C]DCCD (10μM) and F_1-ATPase (0.25mg/ml) were incubated together at 30°C for 10min in 50mM-sodium Mops (4-morpholine-ethanesulphonate), pH 6.5. Expt. B: [^{14}C]Nbf-Cl (100μM) and F_1-ATPase (0.5mg/ml) were incubated together at 30°C for 30min in 0.2M-sucrose/2mM-EDTA/10mM-triethanolamine, pH 7.5. DCCD and Nbf-Cl were added as ethanolic solutions. ATPase activity was assayed at pH 8.5 (see the Methods section). Results cited are means of at least six measurements in each case.

	Inhibition of ATPase (%)	Reagent incorporated (mol/mol of F_1-ATPase)	Proportion of ^{14}C radioactivity in β-subunit (%)
Expt. A (DCCD)			
Normal F_1-ATPase	55	0.82	96
uncD412 F_1-ATPase	54	0.77	88
Expt. B (Nbf-Cl)			
Normal F_1-ATPase	68	2.60	77
uncD412 F_1-ATPase	69	2.84	77

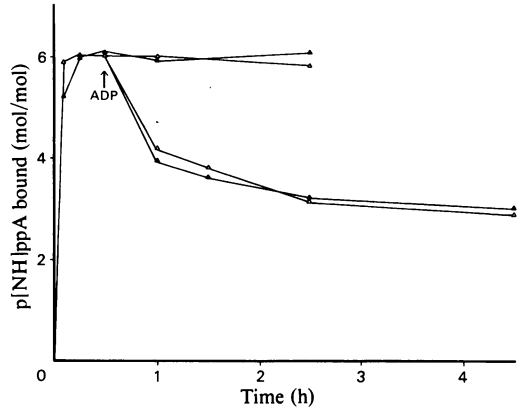

Fig. 3. *Reloading of nucleotide-depleted F_1-ATPase preparations with p[NH]ppA and exchange of reloaded p[NH]ppA with added ADP*
Nucleotide-depleted F_1-ATPase preparations were reloaded with p[NH]ppA as described in the Methods section (upper curves). As indicated by the arrow, ADP (10mM) was added to some samples (after removal of non-bound p[NH]ppA) to study the exchange of reloaded p[NH]ppA (lower curves) (see the Methods section). The data shown are means of triplicate determinations. Similar results were obtained when ATP (1mM) was added instead of ADP (10mM) (*unc*$^+$ enzyme only). ▲, *unc*$^+$; △, *uncD412*.

dominantly by both reagents (Table 2). We conclude that the essential residues with which DCCD and Nbf-Cl react are present in *uncD412* F_1-ATPase and are not environmentally abnormal.

Inhibition and labelling of uncD412 F_1-ATPase by FSBA

Inhibition of normal *E. coli* or *uncD412* F_1-ATPase by FSBA followed apparent first-order kinetics up to 90% inhibition. Initial pseudo-first-order rate constants ($k_{obs.}$) were calculated from slopes of plots of ln (residual ATPase activity) versus time at various FSBA concentrations from 0.05 to 3mM. When $k_{obs.}$ was plotted against FSBA concentration, the curves were hyperbolic (results not shown). The data suggest that FSBA binds reversibly before irreversible inactivation, as described previously by Zoller & Taylor (1979) for FSBA inactivation of cyclic AMP-dependent protein kinase. If this is the case, plots of $1/k_{obs.}$ against 1/[FSBA] should be linear and permit determination of k_i $[=(k_{-1}+k_2)/k_{+1}]$, a Michaelis-type constant for reversible formation of F_1-ATPase·FSBA complex, and of k_2 (rate constant for irreversible-inactivation step) as described by Zoller & Taylor (1979). The values of k_i calculated from such reciprocal plots were: normal F_1-ATPase, 2.60mM; *uncD412* F_1-ATPase, 0.22mM. The values of k_2 calculated were: normal F_1-ATPase, 0.0114min^{-1}; *uncD412* F_1-ATPase, 0.0046min^{-1}.

In labelling studies with [^{3}H]FSBA, three samples of normal (*unc*$^+$) enzyme, which had been inactivated by [^{3}H]FSBA by 40–45%, were found to contain 3.45, 3.59 and 4.67 mol of [^{3}H]FSBA/mol of F_1-ATPase respectively. Two samples of *uncD412* F_1-ATPase that had each been inactivated by 45% were found to contain 3.55 and 4.78mol of [^{3}H]FSBA/mol of F_1-ATPase respectively. Thus, although the data obtained were variable, no clear difference between *uncD412* and normal F_1-ATPase was seen. The data suggest that the labelling of *E. coli* F_1-ATPase by FSBA was less specific than that reported for ox heart mitochondrial F_1-ATPase by Esch & Allison (1978, 1979).

Reloading of nucleotide-depleted uncD412 F_1-ATPase with p[NH]ppA and exchange of reloaded p[NH]ppA with added ATP or ADP

The data in Fig. 3 show that nucleotide-depleted F_1-ATPase from both normal and *uncD412* mutant

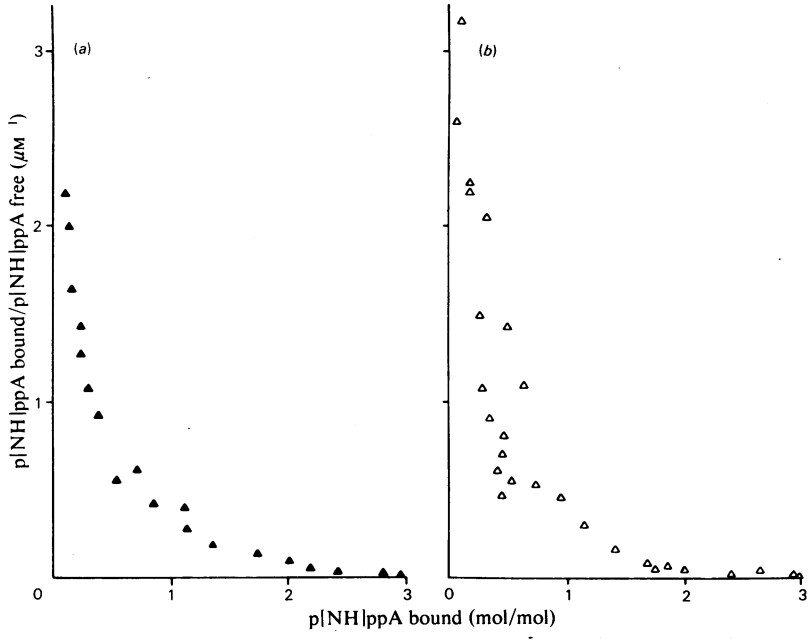

Fig. 4. *Binding of p[NH]ppA to native F_1-ATPase preparations in the presence of Mg*
F_1-ATPase was incubated with p[NH]ppA in 50mM-Tris/H_2SO_4/0.5mM-EDTA/2.5mM-$MgSO_4$, pH 7.5, for 2h at room temperature, then passed through centrifuge columns to separate bound and free p[NH]ppA. Each point is the mean of quadruplicate determinations made on a given day. (*a*) *unc*$^+$ (normal) F_1-ATPase; (*b*) *uncD412* F_1-ATPase.

strains of *E. coli* bound a maximum of six mol of p[NH]ppA/mol of F_1-ATPase. Three of these sites were non-exchangeable when ADP was added, and three of the sites were exchangeable with added ADP. Therefore both *uncD412* and normal F_1-ATPase from *E. coli* resembled ox heart mitochondrial F_1-ATPase, which was shown by Cross & Nalin (1982) to contain three non-exchangeable adenine-nucleotide-binding sites (corresponding to the endogenous or 'tightly bound' nucleotide sites) and three exchangeable adenine-nucleotide-binding sites.

Binding of adenine nucleotides to exchangeable sites in native uncD412 F_1-ATPase in the presence of Mg^{2+}

The binding of p[NH]ppA to the native (i.e. not nucleotide-depleted) *uncD412* F_1-ATPase was compared with the binding to native normal F_1-ATPase. Both enzymes showed maximally three binding sites for p[NH]ppA. The data are presented as Scatchard plots in Fig. 4, and apparent negative co-operativity between the sites is evident. In both enzymes the K_d at the first site was about 0.3μM and the K_d at the third site was about 20μM. These data are similar to those presented previously by Cross & Nalin (1982) for ox heart mitochondrial F_1-ATPase except that the K_d for binding of p[NH]ppA at the first site in *E. coli* F_1-ATPase appeared higher than that in the ox heart enzyme. ADP binding was also measured. For normal F_1-ATPase we found $n=1.99$ and $K_d=3$ μM at both sites (results not shown) and for *uncD412* F_1-ATPase, $n=2.04$ and $K_d=4.15$ μM. When non-radioactive ADP (1mM) was added together with 25μM-radioactive p[NH]ppA in the presence of Mg^{2+}, the amount of p[NH]ppA bound was decreased from 1.7 to 0.35 mol/mol of F_1-ATPase in *uncD412* and from 1.98 to 0.14mol/mol in normal enzyme, showing that ADP and p[NH]ppA competed for the same sites in the presence of Mg^{2+} in both enzymes. It should be pointed out that although only two sites for ADP were seen here, there is evidence that a third MgADP site is filled in normal *E. coli* F_1-ATPase at high ionic strength or high Mg^{2+} concentration (Lunardi *et al.*, 1981).

Binding of adenine nucleotides to exchangeable sites of native uncD412 F_1-ATPase in the absence of Mg^{2+}

Scatchard plots of the binding of p[NH]ppA to *uncD412* and normal F_1-ATPase in the absence of Mg^{2+} were linear with $n=1.2$ and $K_d=160$ μM (results not shown). In both enzymes, aurovertin (10μM) decreased K_d to ~100μM without affecting n significantly. *uncD412* F_1-ATPase bound ADP (in the absence of Mg^{2+}) at one site ($n=0.92$,

$K_d = 23\,\mu M$). When aurovertin ($10\,\mu M$) was present, the K_d was decreased to $13\,\mu M$. In each case a Scatchard plot was linear, and the *uncD412* F_1-ATPase therefore resembled normal F_1-ATPase closely (see Wise *et al.*, 1981). In the absence of Mg^{2+}, when non-radioactive ADP (1 mM) or ATP (1 mM) was added together with radioactive p[NH]ppA ($40\,\mu M$) to *uncD412* or normal F_1-ATPase, binding of p[NH]ppA was decreased by 82–90% in either enzyme, showing that all three adenine nucleotides competed for the same site.

Fluorescence of bound aurovertin

The K_d for aurovertin binding was found to be $1.3\,\mu M$ with *uncD412* F_1-ATPase as against $2.6\,\mu M$ with normal F_1-ATPase in parallel experiments. The fluorescence yield when the two enzymes were saturated with aurovertin was the same. Addition of ADP produced the same level of fluorescence enhancement in both enzymes; $K_{m,ADP}$ was $0.9\,\mu M$ with *uncD412* F_1-ATPase and $1.0\,\mu M$ with normal F_1-ATPase. Therefore *uncD412* was not different from normal F_1-ATPase in intersubunit conformation interaction as measured by aurovertin fluorescence changes (Wise *et al.*, 1981).

Discussion

Catalytic properties of uncD412 F_1-ATPase

The main points of difference between *uncD412* and normal F_1-ATPase that we found were: (*a*) the $V_{max.}$ for hydrolysis of MgATP was 12% of normal at pH 7.5 [confirming our previous finding (Senior *et al.*, 1979*b*)] [one of us (A. E. Senior, unpublished work) has also found that strain AN484 has a low growth yield on limiting (3 mM) glucose which is nevertheless higher than that of a fully uncoupled strain, consistent with the view that ATP synthesis rate is much reduced, but not abolished, in *uncD412* F_1-ATPase]; (*b*) the CaATPase/MgATPase ratio is 15-fold higher than normal; (*c*) the reversible binding of FSBA at the catalytic site was apparently tighter than normal.

Essential residues, aurovertin and efrapeptin inhibition

The labelling experiments with DCCD, Nbf-Cl and FSBA indicated that the essential residues with which these reagents react were present in *uncD412* F_1-ATPase, and no major change in reactivity of these residues was apparent. Efrapeptin, which binds at the catalytic site (Cross & Nalin, 1982), inhibited *uncD412* F_1-ATPase with approximately normal potency. Aurovertin bound with an approximately normal K_d to *uncD412* F_1-ATPase, inhibited normally, and showed normal fluorescence yield once bound.

Nucleotide-binding properties of uncD412 F_1-ATPase

Our data show the total number of adenine-nucleotide-binding sites on normal *E. coli* F_1-ATPase to be six. Of those, three are non-exchangeable and three are exchangeable. Similar findings were reported for ox heart mitochondrial F_1-ATPase (Cross & Nalin, 1982). There is now considerable support for the proposal that the three exchangeable sites are catalytic sites (Esch & Allison, 1978; Grubmeyer & Penefsky, 1981*a*,*b*; Grubmeyer *et al.*, 1982; Cross & Nalin, 1982; Cross *et al.* 1982) and the work presented here supports this view. However, no differences were noted between *uncD412* and normal F_1-ATPase in binding of p[NH]ppA, ADP or ATP at exchangeable or non-exchangeable sites, either in the presence or in the absence of Mg^{2+}. In confirmation of our previous data (Wise *et al.*, 1981) we show here that the presence of Mg^{2+} greatly decreases the K_d for adenine nucleotide binding at the exchangeable sites in *E. coli* F_1-ATPase, and in fact, in the absence of Mg^{2+}, only one exchangeable site can be measured with the technique used.

Conformational properties of uncD412 F_1-ATPase

The measurements of ADP-induced enhancement of bound aurovertin fluorescence showed that the intersubunit conformational interactions which mediate this fluorescence change are operative in *uncD412* F_1-ATPase, and this is confirmed by the fact that aurovertin did modulate the binding affinity for ADP and p[NH]ppA in *uncD412* F_1-ATPase. However, it is quite possible that a partial impairment of intersubunit conformational interaction would have gone undetected in our assays. The catalytic turnover rate of normal F_1-ATPase (30°C, pH 7.5) is about one per 23 ms and that of *uncD412* F_1-ATPase is about one per 200 msec, whereas performance of the aurovertin fluorescence assays requires 1–5 min.

Mechanism of impairment of catalysis in uncD412 F_1-ATPase

The bulk of the evidence presented here showed that the structure of the catalytic sites and binding of adenine nucleotides to the catalytic sites in *uncD412* F_1-ATPase were not abnormal, and that other explanations for the catalytic defect should be considered. One possibility is that the interaction between catalytic sites, which is thought to be required for normal catalytic rates (Grubmeyer *et al.*, 1982; Cross *et al.*, 1982), is impaired in *uncD412* F_1-ATPase. The *uncD412* F_1-ATPase was structurally unstable at high pH and tended to depolymerize, implying that the mutation affects subunit interaction. Also, the decrease in K_i for FSBA inhibition seen in *uncD412* F_1-ATPase could be due to decrease in rate of dissociation of FSBA

from the catalytic site [change in rate of release of product ADP from the catalytic site is known to be a factor in the 'promotion' of catalysis (Grubmeyer *et al.*, 1982)]. However, further work will be required to confirm or deny this possibility.

Comparison of uncD412 F_1-ATPase with catalytically impaired F_1-ATPase preparations from other uncD mutant strains

Senior *et al.* (1983) recently reported isolation and preliminary characterization of F_1-ATPase preparations from four new *uncD* mutant strains of *E. coli*. As discussed in that paper, those four F_1-ATPase preparations were each clearly distinguishable from *uncD412* F_1-ATPase. On the other hand, the *uncD412* F_1-ATPase is quite similar to the KF11 F_1-ATPase as far as can be deduced from the evidence presented (Kanazawa *et al.*, 1980). This latter F_1-ATPase preparation has about 10% of normal MgATPase activity, an enhanced CaATPase activity, and it dissociates into subunits readily. Since the amino acid substitution in KF11 F_1-ATPase did not change the isoelectric point of the β-subunit, it cannot be the same substitution that occurs in *uncD412* F_1-ATPase; however, the two mutations appear functionally equivalent.

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References

Cleland, W. W. (1970) *Enzymes 3rd Ed.* **2**, 1–65

Cox, G. B., Downie, J. A., Langman, L., Senior, A. E., Ash, G., Fayle, D. R. H. & Gibson, F. (1981) *J. Bacteriol.* **148**, 30–42

Cross, R. L. & Kohlbrenner, W. E. (1978) *J. Biol. Chem.* **253**, 4865–4873

Cross, R. L. & Nalin, C. M. (1982) *J. Biol. Chem.* **257**, 2874–2881

Cross, R. L., Grubmeyer, C. & Penefsky, H. S. (1982) *J. Biol. Chem.* **257**, 12101–12105

Esch, F. S. & Allison, W. S. (1978) *J. Biol. Chem.* **253**, 6100–6106

Esch, F. S. & Allison, W. S. (1979) *J. Biol. Chem.* **254**, 10740–10746

Fayle, D. R. H., Downie, J. A., Cox, G. B., Gibson, F. & Radik, J. (1978) *Biochem. J.* **172**, 523–531

Garrett, N. E. & Penefsky, H. S. (1975) *J. Biol. Chem.* **250**, 6640–6647

Gibson, F. (1983) *Biochem. Soc. Trans.* **11**, 229–240

Grubmeyer, C. & Penefsky, H. S. (1981*a*) *J. Biol. Chem.* **256**, 3718–3727

Grubmeyer, C. & Penefsky, H. S. (1981*b*) *J. Biol. Chem.* **256**, 3728–3734

Grubmeyer, C., Cross, R. L. & Penefsky, H. S. (1982) *J. Biol. Chem.* **257**, 12092–12100

Kanazawa, H., Horiuchi, Y., Takagi, M., Ishino, Y. & Futai, M. (1980) *J. Biochem. (Tokyo)* **88**, 695–703

Lunardi, J., Satre, M. & Vignais, P. V. (1981) *Biochemistry* **20**, 473–480

Penefsky, H. S. (1977) *J. Biol. Chem.* **252**, 2891–2899

Satre, M., Bof, M. & Vignais, P. V. (1980) *J. Bacteriol.* **142**, 768–776

Segel, I. H. (1976) *Biochemical Calculations*, 2nd edn., pp. 246–266, John Wiley and Sons, New York

Senior, A. E. & Wise, J. G. (1983) *J. Membr. Biol.* **73**, 105–124

Senior, A. E., Downie, J. A., Cox, G. B., Gibson, F., Langman, L. & Fayle, D. R. H. (1979*a*) *Biochem. J.* **180**, 103–109

Senior, A. E., Downie, J. A., Cox, G. B., Gibson, F., Langman, L. & Fayle, D. R. H. (1979*b*) *Biochem. J.* **180**, 111–118

Senior, A. E., Langman, L., Cox, G. B. & Gibson, F. (1983) *Biochem. J.* **210**, 395–403

Taussky, H. H. & Shorr, E. (1953) *J. Biol. Chem.* **202**, 675–685

Wise, J. G., Latchney, L. R. & Senior, A. E. (1981) *J. Biol. Chem.* **256**, 10383–10389

Wyatt, J. L. & Colman, R. F. (1977) *Biochemistry* **16**, 1333–1342

Zoller, M. J. & Taylor, S. S. (1979) *J. Biol. Chem.* **254**, 8363–8368