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Monoclonal Antibody Modification of the ATPase Activity of *Escherichia coli* F_1 ATPase†

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ABSTRACT: Monoclonal antibodies (mAbs) have been made against each of the five subunits of ECF_1 (α, β, γ, δ, and ϵ), and these have been used in topology studies and for examination of the role of individual subunits in the functioning of the enzyme. All of the mAbs obtained reacted with ECF_1, while several failed to react with ECF_1F_0, including three mAbs against the γ subunit (γII, γIII, and γIV), one mAb against δ, and two mAbs against ϵ (ϵI and ϵII). These topology data are consistent with the γ, δ, and ϵ subunits being located at the interface between the F_1 and F_0 parts of the complex. Two forms of ECF_1 were used to study the effects of mAbs on the ATPase activity of the enzyme: ECF_1 with the ϵ subunit tightly bound and acting to inhibit activity and ECF_1* in which the δ and ϵ subunits had been removed by organic solvent treatment. ECF_1* had an ATPase activity under standard conditions of 93 μmol of ATP hydrolyzed min^{-1} mg^{-1}, cf. an activity of 7.5 units mg^{-1} for our standard ECF_1 preparation and 64 units mg^{-1} for enzyme in which the ϵ subunit had been removed by trypsin treatment. The protease digestion of ECF_1* reduced activity to 64 units mg^{-1} in a complicated process involving an inhibition of activity by cleavage of the α subunit, activation by cleavage of γ, and inhibition with cleavage of the β subunit. mAbs to the γ subunit, γII and γIII, activated ECF_1 by 4.4- and 2.4-fold, respectively, by changing the affinity of the enzyme for the ϵ subunit, as evidenced by density gradient centrifugation experiments. The γ-subunit mAbs did not alter the ATPase activity of ECF_1*- or trypsin-treated enzyme. The α-subunit mAb (αI) activated ECF_1 by a factor of 2.5-fold and ECF_1F_0 by 1.3-fold, but inhibited the ATPase activity of ECF_1* by 30%.

Monoclonal antibodies are being used extensively to study structure–function relationships of the F_1 ATPases of bacteria, plants, and animals [e.g., see Moradi-Ameli and Godinot (1983, 1987), Dunn et al. (1985), and Hadikusumo et al. (1984)]. Included in these studies are examination of cross-reactivity of F_1 ATPases from different organisms [e.g., see

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Archinard et al. (1984), Spitsberg et al. (1985), and Dunn et al. (1985)], determination of the stoichiometry of subunits in the enzyme (Lunsdorf et al., 1984; Tiedge et al., 1985), and discrimination of exposed and buried antigenic sites in the complex (Moradi-Ameli & Godinot, 1987).

The effects of mAbs on the ATPase activity of F_1 have also been explored. Antibodies against the β subunits have been described which inhibit the enzyme activity up to 90% (Dunn et al., 1985). Other mAbs made against the α and β subunits activate F_1 by a small amount, i.e., 25–40% (Hadikusumo et al., 1984; Dunn et al., 1985). Also, an ϵ-specific monoclonal antibody, which increases activity by reversing the inhibition of F_1 by the ϵ subunit, has been described by Dunn and Tozer (1987).

We have recently prepared monoclonal antibodies for a study of the arrangement of subunits in the ECF_1 complex (Gogol et al., 1989b). As a complement to these structural studies, we have examined the effects of our mAbs on ATPase activity. We find that mAbs against the α and γ subunits alter activity in a complex manner related both to their effect on the conformation of the enzyme and to their effect on binding of the ϵ subunit. Studies using enzyme preparations missing the ϵ (and δ) subunit allow us to distinguish between and characterize these different effects.

Materials and Methods

Preparations. ECF_1 was isolated from *Escherichia coli* strain AN1460 by a modification of the method of Wise et al. (1981) described in Gogol et al. (1989a). ECF_1, depleted of δ and ϵ subunits (ECF_1*), was prepared according to Tuttas-Dorschug and Hanstein (1989) using Sephacryl S-300 (Pharmacia) instead of Bio-Gel A 1.5M and also employing the same glycerol-containing buffers used in the ECF_1 purification. ECF_1F_0 was isolated from a sucrose gradient and reconstituted into egg lecithin as previously described (Aggeler et al., 1987).

The purification of monoclonal antibodies, the generation of Fab′ fragments, and the characterization of the antibody preparations are described in Gogol et al. (1989b).

Protease Digestion. Trypsin cleavage of ECF_1 was carried out in the presence of 0.2% LDAO for 2 h at room temperature and at a ECF_1:trypsin ratio of 100:1 according to Gavilanes-Ruiz et al. (1988). ECF_1* was cleaved by trypsin at 0.55 mg/mL in 50 mM Tris-HCl, pH 7.4, 20% glycerol, 2 mM EDTA, 1 mM ATP, 1 mM DTT, and 40 mM 6-aminohexanoic acid, with trypsin at room temperature at a ratio of 1:450 (protease/ATPase, w/w), 1:40, and 1:20 successively. Trypsin cleavage was stopped by addition of a 30-fold excess (w/w) of bovine pancreatic trypsin inhibitor, and samples were analyzed by ATPase activity assay and $NaDodSO_4$–polyacrylamide gel electrophoresis.

Antibody Binding to ATPase. The binding of monoclonal antibodies to ECF_1 and ECF_1F_0 was determined by ELISA as previously described (Gogol et al., 1989b). For some antibodies, the binding to ECF_1F_0, reconstituted in egg lecithin vesicles, was quantitated by the use of radioactively labeled Fab′ fragments, which were prepared according to Gogol et al. (1989b). After reduction of $F(ab')_2$, the concentration of the reducing agent was decreased by passage through a Sephadex G-50 column (1 × 10 cm) equilibrated in PBS (10 mM Na_2HPO_4, pH 7.2, and 150 mM NaCl) containing 50 μM β-mercaptoethanol. *N*-[^{14}C]Ethylmaleimide [NEM, 40 mCi/mmol (New England Nuclear)] was added to a final concentration of 125 μM and reacted for 5 h at room temperature. The ratio of NEM incorporated per Fab′ was 1:1, 0.7:1, and 1.2:1 (mol/mol) for αI, δI, and ϵI, respectively. Increasing concentrations of [^{14}C]NEM–Fab′ in 195 μL of PBS were added to 5 μL of ECF_1F_0 (20 pmol) in 50 mM Tris-HCl, pH 7.5, 5 mM $MgSO_4$, 1 mM DTE, and 10% glycerol. After incubation at room temperature for 1 h, unbound Fab′ was removed by centrifugation at 100000*g* and radioactivity in the pellet was measured in a Beckman LS 7500 scintillation counter.

Antibody Activation of ATPase. The effects of mAbs on activity were measured with 3.8 μg of ECF_1 or 3.5 μg of ECF_1* (10 pmol) in 38 μL of 50 mM Tris-HCl, pH 7.4, 20% glycerol, 2 mM EDTA, 1 mM ATP, 1 mM DTT, and 40 mM 6-aminohexanoic acid. Enzyme was incubated with increasing amounts of monoclonal antibodies or their Fab′ fragments in 137 μL of 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, and 3 mM KCl (TBS) for 1 h at room temperature, in the presence of 1 mg/mL bovine serum albumin. Samples of 100 and 50 μL in the case of ECF_1 and ECF_1*, respectively, were added to an ATP regenerating system to determine the ATPase activity, as described by Lotscher et al. (1984a). The influence of antibodies on the activity of ECF_1F_0, as obtained from sucrose gradients, was measured after incubating 5.3 μg of enzyme (10 pmol) with increasing concentrations of mAbs for 1 h at room temperature in 175 μL of TBS.

Density Centrifugation of ECF_1–mAb. Fifty micrograms of ECF_1 (0.13 nmol) was incubated at room temperature for 3 h with a 6–17-fold excess of mAb in 0.6 mL of the buffer used in the activation experiments, without bovine serum albumin. The ECF_1–mAb complex was applied on a 10–40% sucrose step gradient in the same buffer and run at 4 °C for 18 h in a Beckman SW 50.1 rotor at 40000 rpm. Seven fractions were collected from the bottom of the tubes and assayed for ATPase. The activity-containing fractions were pooled, and the protein was precipitated by addition of sodium deoxycholate and trichloracetic acid to final concentrations of 0.3% and 8%, respectively, and electrophoresed on a 10–18% $NaDodSO_4$–polyacrylamide gel.

Other Methods. Protein concentrations were determined by a modified Lowry procedure (Markwell et al., 1978; Peterson, 1977) for ATPase and by measuring the optical density at 280 nm for the antibodies and their fragments [E_{280}(0.1%, 1 cm) = 1.3–1.5]. Samples for $NaDodSO_4$ electrophoresis were dissolved in 2% $NaDodSO_4$, 5% glycerol, 50 mM DTT, and 0.12 M Tris-HCl (pH 6.8); 1.5-mm-thick slab gels were run as described by Laemmli (1970) with a 3% acrylamide stacking gel and a 10% or 10–18% separating gel. Staining with Coomassie brilliant blue R and destaining were carried out according to Downer et al. (1976).

Results

Range of Activities of ECF_1 ATPase. ECF_1 is purified in our laboratory by a modification of the method of Wise et al. (1981) described in Gogol et al. (1989a). This procedure yields an enzyme with a low ATPase activity, typically 7–8 μmol of ATP hydrolyzed min^{-1} (mg of protein)$^{-1}$ when assayed at 37 °C, pH 7.6, and at a protein concentration of 10 μg/mL. This basal activity can be increased 3-fold by dilution, an effect caused by release of the ϵ subunit from the enzyme (Laget & Smith, 1979; Sternweis & Smith, 1980). Trypsin cleavage increases the ATPase activity of our ECF_1 preparation to 64 units/mg (Gavilanes-Ruiz et al., 1988) (Table I), an effect also attributed to removal of the ϵ subunit. The highest turnover rates are obtained when LDAO is added to the assay buffer, as reported previously (Lötscher et al., 1984b). With enzyme isolated from *E. coli* strain AN1460, we routinely obtain activities of 150–160 units/mg in the presence of 0.5% LDAO (Table I), an activation of more than 20-fold over the

Table I: ATPase Activities (units/mg)[a]

	no LDAO	+LDAO	n
ECF_1	7.5 ± 1.7	154 ± 4	10
$ECF_1(t)$	64 ± 6	122 ± 14	3
ECF_1*	93 ± 6	182 ● 9	8
ECF_1F_0	17 ± 1	65 ● 8	9

[a] ECF_1. Free ECF_1, separated form ECF_0 ($\alpha_3\beta_3\gamma\delta\epsilon$); $ECF_1(t)$, ECF_1, trypsinized to remove δ and ϵ subunits and cleave α(N-terminal), β(C-terminal), and $\gamma(\alpha'_3\beta'_3\gamma'\gamma'')$; ECF_1*, ECF_1, depleted of δ and ϵ subunits ($\alpha_3\beta_3\gamma$); ECF_1F_0, $\alpha_3\beta_3\gamma\delta\epsilon ab_2c_{12}$. n is the number of determinations on different fractions or different preparations of enzyme.

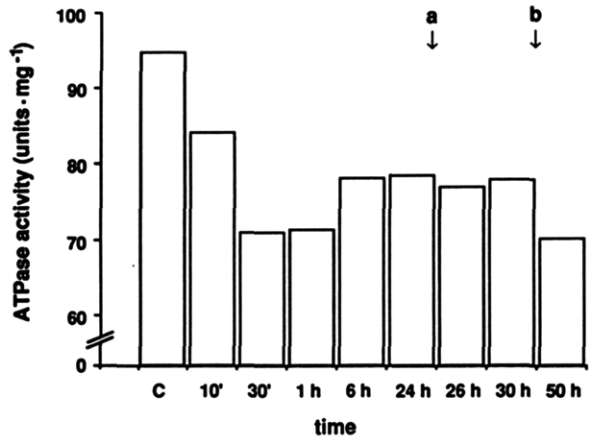

FIGURE 1: Effect of trypsin cleavage of ECF_1* on ATPase activity. ECF_1, depleted of δ and ϵ subunits (0.55 mg/mL), was cleaved with trypsin at a ratio of 1:450 (protease/ATPase, w/w), increasing the trypsin concentration as indicated by the arrows to 1:40 (a) and 1:20 (b). Fifty-microliter samples were taken before the proteolysis (control) and after the indicated times of proteolysis, and 1.5 and 15 μg of bovine pancreatic trypsin inhibitor was added in the case of low and high trypsin concentrations, respectively. The ATPase activity was measured at 2 μg/mL.

basal activity. The effect of LDAO is due in part to an alteration in the binding of ϵ but also appears to involve a direct effect on the catalytic site.

Tuttas-Dorschug and Hanstein (1989) have recently described a purification method involving organic solvents that yields an ECF_1 preparation (ECF_1*) devoid of δ and ϵ subunits. The activity of enzyme prepared in this way was 93 ± 6 units/mg in our assay system, and this was raised to 182 ± 9 units/mg when LDAO was added.

The difference in activity between ECF_1 treated with trypsin to remove ϵ and ECF_1* in which the ϵ subunit was removed by organic solvent was unexpected and intriguing. It suggested that the activation of ECF_1 by trypsin represented the sum of two or more effects: one, an increase in activity by removal of the ϵ subunit; the second, an inhibition caused by cleavage of one of the other subunits, i.e., α, β, or γ. Figure 1 shows the effect of trypsin cleavage on the activity of ECF_1* using a ratio of protease to enzyme of 1:450 for 24 h followed by 1:40 and then 1:20 to ensure complete digestion of the three subunits. Figure 2 follows the cleavage of the γ subunit (panel A) and the α and β subunits (panel B) over the same time course and concentration increases in trypsin. The activity of ECF_1* was reduced initially by trypsin treatment as a consequence of cleavage of the α subunit; activity was then partly restored with cleavage of the γ subunit, but then became inhibited to the same level as trypsin-treated ECF_1 with the cleavage of the β subunit.

Binding of Monoclonal Antibodies to ECF_1 and ECF_1F_0. Monoclonal antibodies against all of the subunits of ECF_1 have been obtained by screening first for binding to intact ECF_1

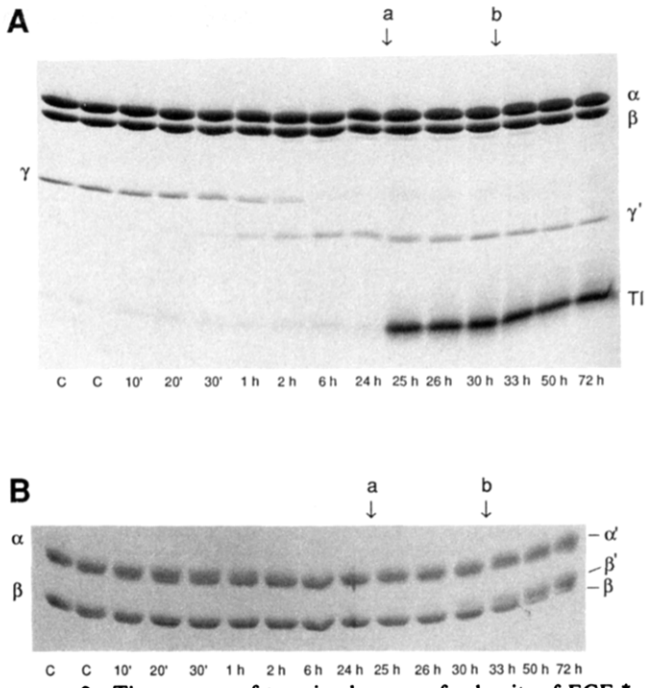

FIGURE 2: Time course of trypsin cleavage of subunits of ECF_1*. Fifty-microliter samples were taken after the indicated times as described in Figure 1, and 18 (A) and 6 μg of ATPase (B) was applied on a 10–18% and a 10% $NaDodSO_4$–polyacrylamide gel, respectively. TI, bovine pancreatic trypsin inhibitor.

Table II: Interaction of Monoclonal Antibodies with ATPase[a]

hybridoma clone no.	antibody designation	reactivity to ECF_1	reactivity to ECF_1F_0	activity (%) of ECF_1	activity (%) of ECF_1F_0
15C3A-H7	αI	+++++	+++++	250	130
7A8-A7	αII	+++++	++++	100	100
7E3-F2	βII	+++++	+++++	100	100
5C1	βIII	+++	+++	ND[b]	ND
3D6-C11	γI	+++++	++++	100	100
8A11-C1	γII	+++++	+	440	100
2A8-F3	γIII	+++++	–	240	ND
11C4	γIV	++++	–	ND	ND
1F7	γV	+++++	++	ND	ND
5A10(1)-C11	δI	+++++	–	100	ND
5A3-C11	ϵI	+++++	–	70	ND
13A7-E9	ϵII	+++++	+	90	ND
6H7	ϵIII	+++++	++++	ND	ND

[a] Antibody binding to ECF_1 and ECF_1F_0 was determined by ELISA with 10 μg/mL ECF_1 and 14 μg/mL ECF_1F_0, respectively, and increasing dilutions of antibody culture media. The absorbance at 410 nm was measured after enzymatic reaction of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) by horseradish peroxidase conjugated to secondary sheep anti-mouse antibody. The activity of ECF_1 at 2.2 μg/mL is 15–16 units/mg (100%); the activity of ECF_1F_0 is 18 units/mg (100%). [b] Not determined.

in ELISA and then by Western blotting, both to establish that the mAbs bind to $NaDodSO_4$-denatured enzyme and to identify the specific subunit involved. Table II summarizes the results of ELISA experiments with each of our mAbs against ECF_1. Two of the mAbs (αI and βII) reacted equally well with both ECF_1 and ECF_1F_0 in ELISA tests: one mAb (γI) reacted with both ECF_1 and ECF_1F_0 but less well with the membrane-bound enzyme; four mAbs bound well to ECF_1 but not to ECF_1F_0.

Binding of many of our mAbs to native ECF_1 (including αI, γI, γII, δI, and ϵI) has been confirmed by immunocryoelectron microscopy (Gogol et al., 1989b), by sedimentation analysis, and by activity effects (see later). Similarly, the binding of αI but not δI or ϵI mAbs to ECF_1F_0 has been confirmed by labeling experiments in which the antibody was

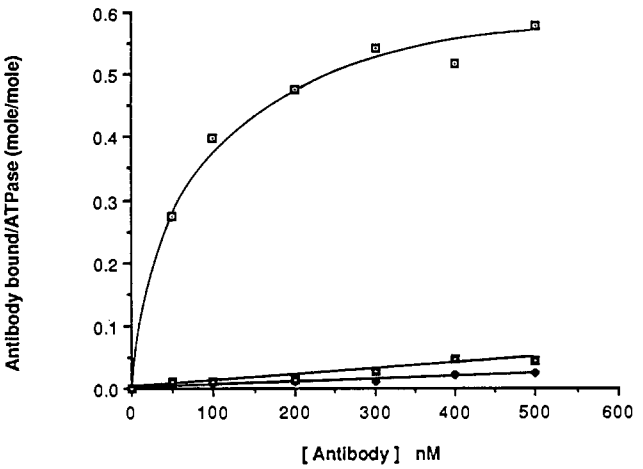

FIGURE 3: Quantitation of antibody binding to ECF_1F_0. ECF_1F_0 (100 nM) was reconstituted in egg lecithin vesicles and incubated with 50–500 nM [^{14}C]NEM–Fab′ for 1 h at room temperature, and radioactivity bound to the pellet after 1-h centrifugation was determined. The Fab′ fragments were derived from αI [(□) 76 000 cpm/nmol], δI [(♦) 50 000 cpm/nmol], and εI (■) 92 000 cpm/nmol].

converted to the Fab′, and modified with [^{14}C]NEM. Thus, we were able to bind up to 0.6 mol of [^{14}C]NEM–αI Fab′ to membranous ECF_1F_0 when the enzyme fraction was reacted with a 5-fold excess of Fab′ to enzyme molecule and then bound and free antibody separated by centrifugation (Figure 3).

Less than optimal binding of the anti-α Fab′, i.e., there could be up to 3 mol bound/mol of ECF_1F_0, is probably due to steric effects. Electron microscopy of the ECF_1F_0 vesicles showed them to contain closely packed enzyme as well as some aggregated protein not incorporated into lipid vesicles (result now shown). Attempts to obtain vesicles with only a few protein complexes to optimize binding measurements, and for imaging of side views of antibody decorated mAbs, have so far failed.

Effect of the mAbs on the ATPase Activity of ECF_1. The effects of the various mAbs on the ATPase activity of ECF_1 were examined with enzyme at a protein concentration of 2 μg/mL, at which the basal activity was 14–16 μmol of ATP hydrolyzed min^{-1} (mg of protein)$^{-1}$. Results are summarized in Table II. Three mAbs, αI, γII, and γIII, activated ECF_1 by 2.5-, 4.4-, and 2.4-fold, respectively; one mAb, εI, inhibited ECF_1 by 30%; the remaining mAbs had no effect on activity.

The concentration dependence of activation of the enzyme by αI, γII, and γIII and the inhibition of activity by εI are shown in Figure 4. The activation by αI occurred over a range of mAb to ECF_1 concentrations of between 1:1 and 5:1 on a molar basis. The Fab′ fragment obtained from the αI mAb was equally effective in activating the ECF_1 complex (also shown in Figure 4). Maximal activation occurred at concentrations of Fab′ shown by electron microscopy studies to result in the binding of three Fab′ fragments per enzyme complex [see Gogol et al. (1989b)]. It should be noted that the concentration dependence of the activation by the αI mAb is slightly sigmoidal. This can be explained by multiple effects of this mAb on the enzyme (see later).

Our electron microscopy studies indicate that ECF_1 can bind several mAbs at once: for example, an enzyme decorated with as many as five monoclonal antibodies (three to α, one to γ, one to δ) has been visualized (Gogol et al., 1989b). It was interesting, therefore, to examine the effect of antibodies added in combination on the ATPase activity of the enzyme. As shown in Figure 5, the activating effects of the anti-α mAb and the anti-γ mAbs were additive. The combination of αI mAb and γII mAb increased the ATPase activity of ECF_1 to

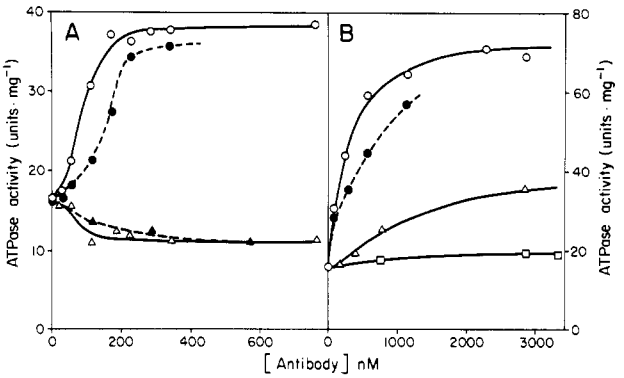

FIGURE 4: Effects of the various mAbs on ATPase activity of ECF_1. The inhibition or activation of ATP hydrolysis of native, purified ATPase (3.8 μg in 175 μL, 57 nM) with the indicated concentrations of antibodies or antibody fragments was measured after incubation for 1 h at room temperature, as described in an ATP-regenerating system (1 unit = 1 μmol of ATP hydrolyzed/min). (A) Activation of ECF_1 by αI immunoglobulin (○) and Fab′ fragment (●); inhibition of ECF_1 by εI immunoglobulin (△) and Fab′ fragment (▲). (B) Activation of ECF_1 by the immunoglobulin of γI (□), γII (○), and γIII (△), and by the Fab′ fragment of γII (●).

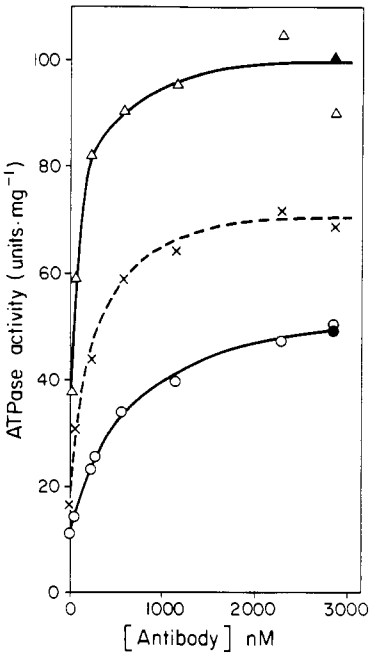

FIGURE 5: Effects of combinations of monoclonal antibodies on the ATPase activity of ECF_1. 3.8 μg of ECF_1–ATPase was incubated with the indicated concentrations of γII for 30 min after a preincubation with 10 μg of εI (○) or 10 μg of αI (△), respectively, for 30 min at room temperature. The sequence of the addition of the two antibodies were reversed: incubation with 75 μg of γII followed by 10 μg of εI (●) or 10 μg of αI (▲). As a control, the activation of the ATPase activity by γII alone was used (×).

90 μmol of ATP hydrolyzed min^{-1} (mg of protein)$^{-1}$, an activity comparable to that of ECF_1*. This activation by the two mAbs in concert was independent of the order of addition of the two antibodies. The combination of γII mAb and εI mAb is also shown in Figure 5. The effects of these two antibodies was also additive, with the anti-ε mAb reducing the activation due to the anti-γ mAb. The combined effect of the anti-γ and anti-ε mAbs was independent of the order of their addition. Anti-ε mAb failed to affect the activation induced by the anti-α mAb. This cannot be explained by a failure of the ε subunit to bind to ECF_1 in the presence of the anti-α mAb. Complexes of ECF_1 with Fab′ fragments from anti-α and anti-ε mAbs have been examined by cryoelectron microscopy (Gogol et al., 1989b). Averaged images of the ECF_1–anti-α, anti-ε–Fab′

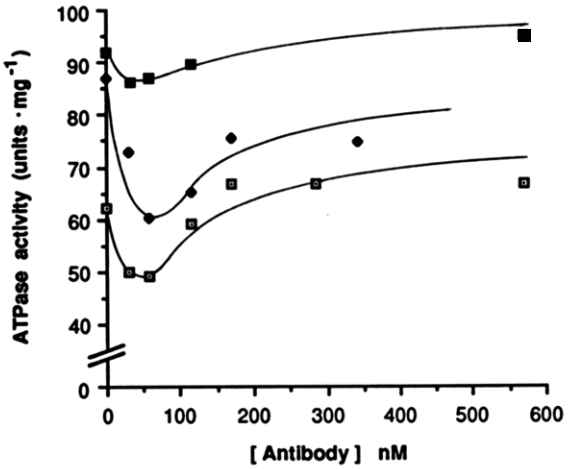

FIGURE 6: Effects of ECF_1-activating antibodies on ATPase, depleted of δ and ϵ subunit. 3.5 μg of δ- and ϵ-free ATPase was incubated for 1 h at room temperature with the indicated concentrations of αI and γII antibodies, respectively. Influence on ATPase activity of γII on ECF_1* (■) and αI on ECF_1* (♦) and on trypsinized ECF_1 (□).

complex demonstrate the presence of four Fab′ fragments (three from α and one from the ϵI mAb) [Figure 9A of Gogol et al. (1989b)] with the anti-ϵ antibody bound near a β subunit.

Combinations of three different mAbs were also tested. The addition of anti-δ or anti-ϵ mAbs in concert with both anti-α and anti-γ mAbs gave the same activity as obtained with anti-α plus anti-γ mAbs only. There was no further activation but, importantly, no inhibition of ATPase activity with addition of a total of five mAbs.

Effect of the α mAb on the Activity of ECF_1F_0. αI is the only mAb in our collection which both affects the activity of ECF_1 and binds to ECF_1F_0. This mAb proved to be an activator of ECF_1F_0 at low concentrations. In several different experiments, the ATPase activity was 1.3-fold higher when 1 mol of mAb per mole of enzyme was added. This activating effect of the αI mAb may result from an altered binding of the ϵ subunit. Trypsin treatment of ECF_1F_0, which cleaves the ϵ subunit (Gavilanes-Ruiz et al., 1988), also activates the enzyme, in this case by 1.7-fold.

Effect of mAbs on the ATPase Activity of ϵ-Deficient ECF_1 (ECF_1).* One obvious explanation for the activation of ECF_1 by the anti-α and anti-γ mAbs is that they affect the binding of the inhibitory ϵ subunit. If so, the antibodies should not affect the activity of ECF_1* (which is already activated because of the removal of the ϵ subunit). The concentration dependencies of the effect of αI and γII on ECF_1* are shown in Figure 6. The activating effect of both antibodies is lost on reaction with ECF_1*. Surprisingly, the αI mAb inhibited the ATPase activity of ECF_1*, the greatest inhibition being at low ratios of mAb to enzyme, with some but not full regeneration of activity by addition of higher amounts of antibody. The same unusual concentration dependence was obtained when the Fab′ generated from the αI mAb was used. The data suggest that inhibition is greatest with one anti-α mAb bound per ECF_1, but with part release of this inhibition when two or three mAbs are bound per enzyme molecule.

The effect of the α and γ antibodies on trypsin-treated enzyme was also examined. None of the antibodies activated trypsin-treated ECF_1, but instead the αI mAb inhibited the ATPase activity of the preparation as it did for ECF_1* (Figure 4).

From the above results, the αI mAb can be expected to have two opposing effects on ECF_1; activation due to altered binding of the ϵ subunit and inhibition caused by binding of the mAb to the α subunit. These competing effects, with inhibition occurring at low concentrations and then release of the ϵ subunit inhibition at higher concentrations of mAb, would explain the sigmoidal curves in Figure 5.

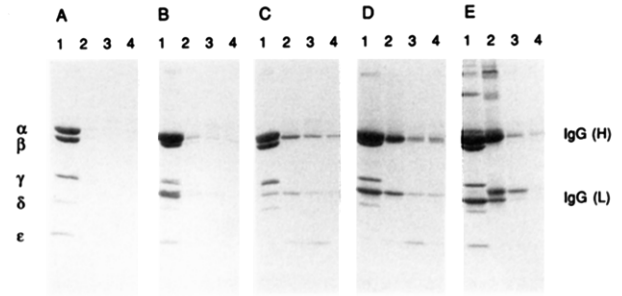

FIGURE 7: Sucrose gradient centrifugation of ECF_1–antibody complexes. ECF_1 (0.13 nmol) was incubated with no antibody (A), 0.8 nmol of αI (B), 2.2 nmol of γII (C), 0.8 nmol of αI and 2.2 nmol of γII (D), and 2.2 nmol of γI (E) and applied on a 10–40% sucrose step gradient. The pooled ATPase activity containing fractions and the three top fractions, 5–7, of the sucrose gradient were applied after concentration on lane 1 and lanes 2–4, respectively, of a 10–18% $NaDodSO_4$–polyacrylamide gel. IgG(H) and IgG(L) indicate the positions of the heavy and light chains of immunoglobulins.

Sucrose Gradient Centrifugation of ECF_1–mAb Complexes. Further evidence that the activating mAbs αI and γII increase ECF_1 activity by altering the binding of the ϵ subunit to the complex was sought in sedimentation experiments. The ϵ subunit binds to ECF_1 with an inhibition constant of around 2–10 nM (Dunn et al., 1990; Sternweis & Smith, 1980; Dunn, 1982). When ECF_1 was sedimented through a sucrose gradient at a protein concentration of 220 nM added protein, the ϵ subunit remained associated with the complex, as shown in Figure 7A. Reaction of ECF_1 with αI, which activates the enzyme by 2-fold, did not release significant amounts of the ϵ subunit based on the sucrose gradient assay (Figure 7B). The three anti-γ mAbs behaved differently from each other. mAb γI, which has no effect on activity, did not release the ϵ subunit. The γII and γIII mAbs each released the ϵ subunit to an extent proportional to their activating effect; i.e., there was some ϵ still bound with γIII and essentially none with γII. The combination of αI and γII also released the ϵ subunit from ECF_1 completely. This release of the ϵ subunit must reflect an altered affinity of the core complex for ϵ caused by the antibody, and a dissociation effect of the sucrose gradient step. mAb binding by itself did not dissociate the ϵ subunit as indicated by the effect of anti-ϵ mAbs on the ECF_1-γII complex (Figure 5).

Discussion

The ECF_1 complex is made up of five different subunits, α, β, γ, δ, and ϵ, with the roles of some of these subunits more clearly defined than others (Senior, 1988). Thus, the catalytic sites appear to be located on the β subunits, possibly at the interface between α and β subunits (Schafer et al., 1985; Cross et al., 1987). The α subunits are known to be involved in cooperative ATPase activity, with mutations in several sites reducing the cooperativity between catalytic sites without affecting single-site turnover (Wise et al., 1984; Senior, 1988). The role(s) of the γ subunit remain(s) undefined. One suggestion is that this subunit is the link between, and the gate for, the proton channel (Kagawa, 1978). The δ subunit appears to link F_1 to F_0 as a part of the stalk between these two parts of the complex. Finally, the ϵ subunit has been found to act as an inhibitor of ATPase activity, at least in isolated ECF_1.

In studies described here, mAbs to each of the different subunits have been examined for their reaction with both ECF_1 and ECF_1F_0 and for effects on ATPase activity. The results

provide information on the topology of the ATP synthase, the role of the ϵ subunit as an inhibitor of ATPase activity in ECF_1, and the importance of structural rearrangements in enzymatic activity.

Antibodies to the α and β subunits reacted with both isolated ECF_1 and the membrane-bound form of the complex. One of the mAbs to the γ subunit and a mAb to the ϵ subunit bound to ECF_1 in solution and as associated with F_0. mAbs to the γ, ϵ, and our single mAb to δ reacted with ECF_1 but not ECF_1F_0. The epitopes for these mAbs are probably involved in subunit–subunit interactions between the F_1 and F_0 parts. We have shown that ECF_1 is linked to the membrane-intercalated part by a narrow stalk approximately 45 Å long (Gogol et al., 1987). Thus, there is access to the stalk region, based on size considerations, both by proteases and by antibodies. Other antibodies to the γ and ϵ subunits and an anti-δ mAb (Dunn & Tozer, 1987) have been found to react with the intact F_1F_0.

There is considerable evidence that the ϵ subunit is an inhibitor of ECF_1. The ϵ subunit binds with a K_i of around 2 nM (Dunn et al., 1990). Dilution to a protein concentration low enough to release the ϵ subunit activates the enzyme as much as 3-fold (Laget & Smith, 1979). The ATPase activity of ECF_1 is also increased 8-fold by protease digestion in a time course that follows the cleavage and release of the ϵ subunit (Gavilanes-Ruiz et al., 1988). In this study, we have compared the ATPase activity of ECF_1*, a preparation of the enzyme from which the ϵ subunit has been released by organic solvent treatment, with that of native enzyme. The difference in activities of these preparations is 12-fold: Under identical assay conditions, ECF_1* had an activity of 93 ± 6 units/mg, the trypsin-treated enzyme 64 ± 6 units/mg, and native enzyme 7.5 ± 1.7 units/mg. According to these data, the inhibitory effect of ϵ in ECF_1 is comparable to that of the ϵ subunit in CF_1 (Richter et al., 1984). The lower activation achieved by trypsin treatment is due to multiple effects of subunit cleavage. While removal of ϵ and cleavage of the γ subunit have activating effects, removal of the N-terminus of the α subunit and cleavage of the C-terminus of the β subunit both contribute an inhibition of ATPase activity.

Binding of some of the anti-γ mAbs and the anti-α mAb was found to activate native ECF_1 but not to increase the activity of enzyme from which the ϵ subunit had been removed. mAbs γII and γIII altered the binding of the ϵ subunit as indicated by sucrose gradient centrifugation experiments. These two mAbs bind to the C-terminal region of the γ subunit (M. Gavilanes-Ruiz, R. Aggeler, and R. A. Capaldi, unpublished results). mAb γI, which does not activate ECF_1, did not release the ϵ subunit. This mAb appears to bind to the N-terminal two-thirds of the γ subunit in preliminary epitope mapping experiments. There is evidence from cross-linking studies (Bragg & Hou, 1976, 1980, 1986; Aris & Simoni, 1983) and from direct binding experiments (Dunn, 1982) that the ϵ subunit is bound to γ in the ECF_1. Therefore, the altered affinity of the enzyme for ϵ when selected anti-γ mAbs are bound could be due to induced conformational changes in the γ subunit and/or to competition of the anti-γ mAbs for the ϵ binding site. The anti-α mAb activated ECF_1 without release of ϵ in the sucrose gradient experiments. Nevertheless, activation by this antibody is probably related to ϵ, perhaps shifting it from an inhibitory site without a dramatic alteration in binding affinity. There is no evidence of interaction between the α and the ϵ subunits in ECF_1, so the α subunit may exert its effect on ϵ indirectly, through the γ subunit. LDAO also activates ECF_1, presumably in part by releasing inhibition by the ϵ subunit though the treatment does not release ϵ in sucrose gradient experiments (Lotscher et al., 1984b).

Cooperative conformational changes are obviously important in ATP hydrolysis and ATP synthesis by F_1F_0-type ATPases. Recent experiments with TF_1 show that α and β subunits alone can form an F_1 which shows cooperative ATPase activity (Kagawa et al., 1989; Miwa & Yoshida, 1989), indicating that communication between catalytic sites is propagated across α–β-subunit interfaces, possibly by rotations of these alternating subunits relative to one another. The important TF_1 studies also show that the asymmetry of F_1 ATPase is induced by substrate binding, rather than being due to interaction of the small subunits. The small subunits must respond to and control cooperativity in their roles in coupling ATPase activity or ATP synthesis to the proton channel and as inhibitors of ATPase activity in the case of the ϵ subunit. The inhibitory effect of the anti-α mAbs on ECF_1* could be due to an antibody-induced conformational change in the α subunit or to steric effects which slow nucleotide-induced conformational changes. Cleavage of the α subunit in ECF_1* has the same effect as mAb binding, suggesting that the mAb inhibits by inducing a structural change in the enzyme.

A surprising result is the biphasic nature of the effect of the anti-α mAb on the activity of ECF_1*. The mAb inhibits ATPase activity at low concentrations presumably when 1 mol is bound, but the inhibition is relieved at high concentrations of mAb when the stoichiometry of binding reaches 2–3 mol of αI/mol of ECF_1*. One explanation for this finding is that the enzyme works better when the three α subunits can go through equivalent conformations, as expected for an alternating-site mechanism.

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Localization of a Reactive Exofacial Sulfhydryl on the Glucose Carrier of Human Erythrocytes†

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ABSTRACT: Tryptic digestion studies of the human erythrocyte glucose carrier have shown that a reactive and transport-sensitive exofacial sulfhydryl is located in the carboxy-terminal half of the molecule, corresponding to Cys^{347}, Cys^{421}, or Cys^{429}. In the present studies, the erythrocyte glucose carrier labeled on the exofacial sulfhydryl with bis(maleimidomethyl) ether-L-[^{35}S]cysteine was chemically cleaved, either at tryptophans by *N*-bromosuccinimide or at nonalkylated cysteines by 2-nitro-5-thiocyanobenzoic acid. The resulting fragments were separated by linear gradient polyacrylamide gel electrophoresis, and the labeled fragments were identified by their apparent molecular weight, and by immunoblotting with antibodies to specific regions of the carrier protein. All of the labeled fragments were recognized by an antibody to the carboxy terminus of the carrier, but not by an antibody to a cytoplasmic loop on the C-terminal half of the carrier. The labeled exofacial sulfhydryl was assigned to Cys^{429}, since this is the only residue of the three possibilities which is beyond the expected cleavage sites of the two reagents in the carrier sequence. These results concur with the predictions of hydropathy analysis and will be relevant for studies of how modification of this sulfhydryl affects carrier function, particularly since several other known carrier isoforms lack a corresponding cysteine.

The facilitative glucose carrier of human erythrocytes has an exofacial sulfhydryl which is reactive with a variety of sulfhydryl reagents which either do not penetrate the cell membrane (Batt et al., 1976; Roberts et al., 1982; May, 1987, 1989a) or do so very slowly (May, 1989b). In addition to its exposure on the cell surface, this group has several features which make it potentially useful in the study of structure–function relationships of the carrier: (1) it is exposed when the carrier is in a conformation ready to accept substrate from without, but not from within the cell (Krupka, 1985; May, 1989a,b), (2) it is not required for substrate binding (Krupka & Devés, 1986; May, 1989a), (3) its reaction may or may not affect the ability of the carrier to subsequently change conformation depending on the sulfhydryl reagent used (May, 1989a,b), and (4) its reaction with low concentrations of *N*-ethylmaleimide has no apparent effect on transport at all (May, 1989c).

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