Assembly of the Catalytic Unit of the *Escherichia coli* Membrane ATPase in Vitro Requires the γ Chain†

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ABSTRACT: Freezing the purified F_1 portion of the *E. coli* membrane ATPase at high ionic strength in the presence of KNO_3 reversibly inactivated the enzyme and dissociated the two larger α and β subunits to monomers. After cold inactivation, the F_1 subunits were separated into two fractions by hydroxylapatite chromatography. One fraction contained chiefly the α and β subunits ($\alpha\beta$ fraction), while the other contained α, β, and about a threefold excess of γ and ϵ ($\gamma\epsilon$-rich fraction), by comparison with the native enzyme. Dialysis against MgATP restored full ATPase activity to the $\gamma\epsilon$-rich fraction, but the $\alpha\beta$ fraction remained inactive. Combining the two fractions produced a synergistic restoration of ATPase activity. When the $\alpha\beta$ fraction was present in excess, about threefold more activity was restored than was obtained with the $\gamma\epsilon$-rich fraction alone. Purified δ and ϵ, either separately or together, were ineffective in restoring activity to the $\alpha\beta$ fraction, nor did δ or ϵ affect the reconstitution of the ATPase by the $\gamma\epsilon$-rich fraction. Since the ATPase appears to be fully active without an intact γ subunit, it is probably the *assembly* of the catalytic unit after cold inactivation which explains the requirement for the γ chain for the restoration of ATPase activity after cold inactivation. Epsilon may or may not be needed along with γ. Subunit γ did not act catalytically in the assembly of ECF_1 but was required in a definite amount relative to α and β. The excess γ remained active in the assembly of the ATPase after the α and β chains present in the $\gamma\epsilon$-rich fraction were assembled into active enzyme. The $\gamma\epsilon$-rich fraction reconstituted cold inactivated $\alpha\beta$-ATPase prepared by the digestion of EC-F_1 with trypsin which removes the γ, δ, and ϵ subunits. Furthermore, while $\alpha\beta$-ATPase is insensitive to inhibition by the ϵ subunit, it regained full sensitivity to the inhibitory subunit after reconstitution with the $\gamma\epsilon$-rich fraction. Finally, it was observed that the $\alpha\beta$ fraction became enormously viscous after dialysis against MgATP at room temperature, but the $\gamma\epsilon$-rich one did not.

The F_1 portion of the proton-translocating ATPase[1] is a peripheral membrane protein which is composed of five types of subunits referred to as α, β, γ, δ, and ϵ (Racker, 1976). The two larger α and β subunits are sufficient for catalyzing ATP hydrolysis (which of course is uncoupled from vectorial proton transport in the absence of the F_0 portion of the enzyme), since digestion of chloroplast F_1 with trypsin removes the three smaller subunits without decreasing ATPase activity (Nelson et al., 1974). While the three smaller polypeptides of EC-F_1 are apparently not required for ATPase activity, two of them have recently been purified to homogeneity and shown to perform specific, noncatalytic functions in the holoenzyme (Smith and Sternweis, 1977; Smith et al., 1975). The attachment of F_1 to F_0 showed an absolute requirement for δ and about one δ per F_1 was sufficient (Smith and Sternweis, 1977). The purified ϵ subunit specifically inhibits EC-F_1 ATPase activity, indicating that ϵ is a regulatory subunit (Smith et al., 1975; Smith and Sternweis, 1977) like the inhibitory subunits of the mitochondrial and chloroplast enzyme (Pullman and Monroy, 1963; Nelson et al., 1972). Although a specific function has not yet been found for the γ polypeptide, it is required for energy transduction by the complete proton-translocating ATPase. McCarty and Fagan (1973) showed that the γ subunit of the chloroplast F_1 (CF_1) becomes specifically labeled with NEM when chloroplasts are illuminated indicating illumination causes a conformational change in CF_1 which exposes a reactive group in γ. The light-dependent reaction of NEM with γ inhibited photophosphorylation (McCarty and Fagan, 1973).

A promising approach for isolating the three larger subunits of EC-F_1 in a reconstitutive form takes advantage of the cold lability of the enzyme. Exposure of the purified EC-F_1 to low temperature and a high ionic strength reversibly inactivates the enzyme and the inactivation was shown to be due to the dissociation of the subunits from one another (Vogel and Steinhart, 1976; Smith et al., 1976). The principle advantage of cold inactivation is that it probably disrupts subunit interactions without denaturing the individual subunits. Recently Vogel and Steinhart (1976) succeeded in obtaining an apparently homogeneous preparation of the β subunit of EC-F_1 by cold inactivation. Moreover, by mixing the β with a subfragment containing α, γ, and ϵ and diluting in the presence of MgATP, these authors were able to restore about 70% of the original ATPase activity. Here we describe experiments which show that the γ subunit is necessary for the restoration of ATPase activity after cold inactivation. Since γ does not appear to be part of the catalytic site of the ATPase as mentioned above, we conclude that γ is needed for the assembly of the dissociated α and β into the catalytic unit.

Materials and Methods

Purification of EC-F_1. The K12 (λ) strain of *E. coli* (ATCC

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[1] Abbreviations used are: F_1, the portion of the proton-translocating ATPase which is peripheral to the membrane; ECF_1, F_1 from *E. coli*; F_0, the portion of the proton-translocating ATPase that is an integral component of the membrane; α, β, γ, δ, ϵ, separate polypeptides of ECF_1 in order of decreasing size; DTT, dithiothreitol; ATPase, adenosine triphosphatase; ATP, adenosine triphosphate; EDTA, (ethylenedinitrilo)-tetraacetic acid; SED buffer, 50 mM succinate-Tris (pH 6.0), 0.1 mM NaEDTA, and 0.1 mM DTT.

10798) was grown in minimal medium supplemented with 1 μg/mL thiamine and 1% (v/v) glycerol (Smith and Sternweis, 1977). The pH was maintained at about 7 during growth by the continuous addition of NaOH. Cells were harvested during the late logarithmic phase of growth and stored at −80 °C. When EC-F_1 was purified from these cells by the method described previously (Smith and Sternweis, 1977), it lacked the δ subunit which simplified the separation of the other four subunits after cold inactivation. The purified enzyme was highly active hydrolytically (100 U/mg), but did not attach to F_1-depleted membranes unless the purified δ subunit was added. If purified δ was added to the EC-F_1 missing δ, it attached to membrane vesicles and catalyzed energy transducing reactions in the vesicles.

The reason for using EC-F_1 missing δ was to simplify the separation of the subunits after dissociating them from one another.

Cold Inactivation and Reactivation of EC-F_1. The purified enzyme was precipitated four times with ammonium sulfate (65% saturation) to remove glycerol and ATP present in the storage buffer. Then the enzyme was completely inactivated by freezing overnight at −20 °C at a concentration of about 10 mg/mL in 50 mM Tris–succinate, pH 6.0, 1.0 M KCl, 0.1 M KNO_3, 0.1 mM dithiothreitol (DTT), and 1 mM NaEDTA, pH 7.0 (inactivation buffer). After thawing, ATPase activity could be fully restored by overnight dialysis at room temperature against ATPase reconstitution buffer: 50 mM Tris-HCl, pH 7.3, 10% (v/v) glycerol, 0.05 mM NaEDTA, pH 7.0, 1 mM DTT, and 5 mM MgATP (Smith et al., 1976). A partial restoration of ATPase activity after cold inactivation was achieved by decreasing the salt concentration at least twofold by dilution with the reconstitution buffer as reported by Vogel and Steinhart (1976).

Chromatography of Inactive Subunits. About 30 mg of purified enzyme, which was inactivated by freezing in the presence of high salt as described above, was thawed quickly at 23 °C and diluted 10 times with buffer at 4 °C containing 50 mM succinate–Tris, pH 6.0, 0.1 mM NaEDTA, and 0.1 mM DTT (SED). Subsequent operations were carried out at 4 °C. The cold inactivated enzyme was applied to a 1.5-cm OD column containing 4 g (dry weight) of Bio-Gel HTP hydroxylapatite (Bio-Rad Laboratories, Richmond, Calif.) equilibrated with SED buffer. The column was eluted with a 100-mL linear gradient of 0–200 mM potassium phosphate in SED, pH 6.0, and 30-drop fractions were collected. Alternatively, about 10 mg of enzyme was applied to 1 g of hydroxylapatite and eluted stepwise with potassium phosphate in SED, pH 6.0. Ten-milliliter volumes of 50, 100, 150, 200, and 250 mM phosphate were used for elution and five fractions of equal volume were collected for each step.

Molecular weights of the subunit fragments obtained after cold inactivation were estimated by molecular sieve chromatography. About 30 mg of enzyme which had been inactivated as described was applied to a calibrated Sephadex G-200 column (Pharmacia) equilibrated with inactivation buffer. The column was eluted with the same buffer at a flow rate of about 10 mL/h and 30-drop fractions were collected.

Analytical Methods. Protein was determined by a modification of the method of Lowry et al. (1951) using bovine serum albumin as the standard. ATPase activity was measured as described previously (Futai et al., 1974). Sodium dodecyl sulfate electrophoresis was carried out according to Laemmli (1970).

Results

Reversible Cold Inactivation and Reactivation of EC-F_1.

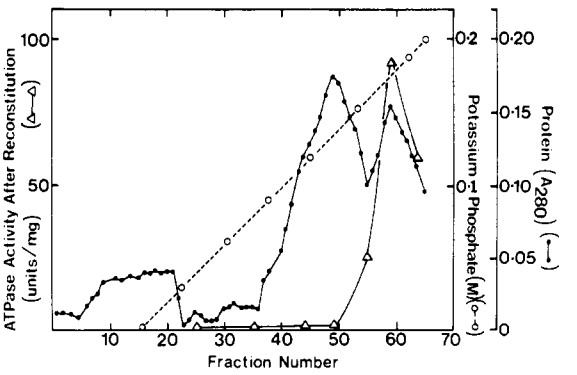

FIGURE 1: Hydroxylapatite chromatography of EC-F_1 after cold inactivation. Fractions from each of the protein peaks were dialyzed overnight at room temperature against reconstitution buffer containing 5 mM MgATP. Before dialysis, all fractions were devoid of activity.

Purified EC-F_1 was completely inactivated by freezing and thawing as described in methods and about 20 mg of enzyme was applied to a calibrated G-200 column equilibrated with inactivation buffer. Elution with the same buffer resulted in two major peaks of protein. The first peak consisted primarily of high molecular weight impurities which were present in small amounts in the purified enzyme as well as some aggregated subunits of EC-F_1. The second peak contained the inactive EC-F_1 fragments which had an apparent molecular weight of about 50 000 and a sedimentation coefficient of 3.5 S. These results imply that the α and β subunits were probably dissociated to monomers by the freezing in high salt. To determine whether ATPase activity could be restored to the enzyme after chromatography on G-200, it was dialyzed against reconstitution buffer (RB) containing various concentrations of MgATP. Dialysis against RB containing 5 mM MgATP restored almost full activity to the cold-inactivated enzyme and this restoration of activity was dependent on the presence of both Mg^{2+} and ATP. No significant restoration of ATPase activity was observed with Mg^{2+} or ATP alone. In addition, the sedimentation coefficient of reactivated enzyme was 12.2 which is the same value that we obtained for the native enzyme indicating that the size and shape of reconstituted and native EC-F_1 were not significantly different in the ultracentrifuge. Furthermore, the reconstituted ATPase which was reisolated by molecular seive chromatography contained all four subunits (α, β, γ, and ε) and with the addition of purified δ subunit became an active coupling factor in the energy transducing reactions in inverted membrane vesicles. The sedimentation coefficient of 3.5 S after cold-inactivation is the same as that of the smallest species noted in the ultracentrifuge when F_1 was dissociated in the cold (Penefsky and Warner, 1965).

Vogel and Steinhart (1976) used dilution instead of dialysis in MgATP to reactivate the ATPase and obtained slightly different results from us. Dilution in MgATP reconstituted about 70% of the original activity and about 30% of the original activity was restored by Mg^{2+} along (Vogel and Steinhart, 1976). The latter result may be due to an incomplete removal of bound nucleotides after cold inactivation.

Chromatography of EC-F_1 Subunits. Purified EC-F_1 was frozen in the inactivation buffer and left at −20 °C overnight to dissociate the enzyme into subunits which are devoid of catalytic activity. The cold-inactivated enzyme was applied to an hydroxylapatite column and eluted with a linear gradient of potassium phosphate. The subunits eluted from the column in two major peaks (Figure 1). The first peak contained primarily the two larger α and β subunits of the enzyme in approximately equal amounts (Figure 2). This will be referred

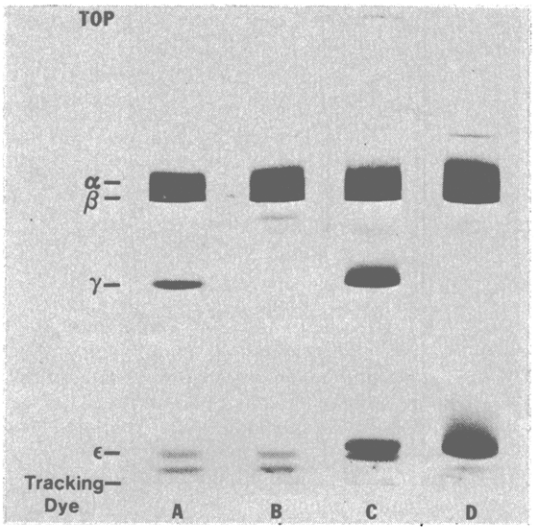

FIGURE 2: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of EC-F_1 before and after cold inactivation and fractionation of subunits on hydroxylapatite. (A) Native enzyme (minus the δ subunit) before cold inactivation; (B) first fraction to elute from the hydroxylapatite column consisting primarily of α and β subunits ($\alpha\beta$ fraction); (C) second fraction to elute from hydroxylapatite column containing α and β subunits and also γ and ϵ polypeptides which are present in excess compared with native enzyme ($\gamma\epsilon$-rich fraction); (D) $\alpha\beta$ fraction to which purified ϵ has been added in excess of that present in the native enzyme. Gel samples were incubated in 1% sodium dodecyl sulfate and 2% mercaptoethanol for 3 min at 100 °C before they were electrophoresed in 11.5% acrylamide gels for about 350 volt-hours and stained with Coomassie blue according to the method of Laemmli (1970). About 21, 16, and 25 μg of protein were applied to gels A, B and C, respectively. Gel D received approximately 27 μg of $\alpha\beta$ fraction to which 19 μg of purified epsilon was added. Since the gels were overloaded, the α and β bands smeared together. When less protein was electrophoresed, α and β bands in all fractions were distinct from one another and stained with approximately equal intensity.

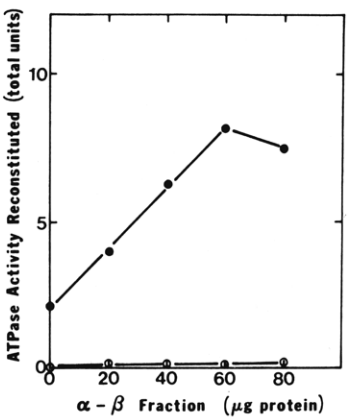

FIGURE 3: Effect of $\gamma\epsilon$-rich fraction on restoration of catalytic activity to $\alpha\beta$ fraction. About 18 μg of $\gamma\epsilon$-rich fraction was incubated with various amounts of the $\alpha\beta$ fraction (●—●) and the mixtures were dialyzed overnight at room temperature against reconstitution buffer. The mixtures were then assayed for ATPase activity as in Futai et al. (1974). No significant amount of ATPase activity was restored to the $\alpha\beta$ fraction in the absence of the $\gamma\epsilon$-rich fraction (O—O). The amount of ATPase activity obtained when the $\gamma\epsilon$-rich fraction was dialyzed by itself is shown by the closed circle on the vertical axis.

to as the $\alpha\beta$ fraction. The $\alpha\beta$ fraction contained some ϵ subunit and some very minor impurities (Figure 2). The second protein peak which eluted at a higher phosphate concentration contained the α and β subunits as well as γ and ϵ (Figure 2). Furthermore, γ and ϵ were present in considerable excess over the amounts seen in the native enzyme. This fraction will be referred to as γ and ϵ rich. On the basis of staining with Coomassie blue, it appears that the ratio of γ or ϵ to α or β in the $\gamma\epsilon$-rich fraction (gel C) is three to four times higher than in the native enzyme (gel A) (Figure 2).

To find out if ATPase activity could be restored to the subunit fractions, they were dialyzed at room temperature against reconstitution buffer containing MgATP. The dialysis procedure restored nearly full activity to the cold inactivated enzyme before it was chromatographed on the hydroxylapatite column. Similarly, nearly full activity was restored to the $\gamma\epsilon$-rich fraction from the hydroxylapatite column since the specific activity of this fraction after reconstitution was almost the same as that of the purified enzyme before cold inactivation. However, the fraction containing only the α and β subunits remained totally devoid of any catalytic activity after dialysis against the reconstitution buffer (Figure 1). Previously Nelson et al. (1974) showed that the α and β subunits of ECF_1 are sufficient for catalyzing ATP hydrolysis since digestion with a protease removed the γ, δ, and ϵ subunits without decreasing ATPase activity. Therefore, since ATPase activity could not be restored to the mixture of α and β subunits unless γ and ϵ were present, it appeared that one or both of these subunits had an essential role in the assembly of the catalytic unit in vitro. The results which follow indicate that the γ subunit is required for the assembly of the catalytic unit in vitro since the purified ϵ subunit by itself was unable to restore activity to the $\alpha\beta$ fraction.

Restoration of ATPase Activity to the $\alpha\beta$ Fraction. If γ were necessary for restoring ATPase activity after cold inactivation, then it might be possible to use the $\gamma\epsilon$-rich fraction to reactivate the $\alpha\beta$ fraction. Figure 3 shows that the $\gamma\epsilon$-rich fraction did reactivate the $\alpha\beta$ fraction which by itself is devoid of any activity after dialysis against MgATP. By titrating a given amount of the $\gamma\epsilon$-rich fraction with the $\alpha\beta$ fraction, the total activity reconstituted increased linearly with increasing amounts of the $\alpha\beta$ fraction until a maximum value was reached (Figure 3). The reconstitution was maximal when the $\alpha\beta$ fraction was present at about a threefold excess over the $\gamma\epsilon$-rich fraction (Figure 3) which agrees with the estimate of the relative degrees of enrichment with γ and ϵ. The demonstration of a synergistic restoration of ATPase activity by the combination of $\alpha\beta$- and $\gamma\epsilon$-rich fractions strongly suggests that the γ subunit is required for reconstituting the ATPase after cold inactivation. The fact that the restoration of activity was essentially 100% rules out the possibility that either of the two fractions contained defective α or β.

Since the ϵ subunit of EC-F_1 is available in homogeneous form (Smith and Sternweis, 1977), we tried ϵ by itself and found that it was insufficient to restore ATPase activity to the $\alpha\beta$ fraction.

ϵ did not restore any activity to the $\alpha\beta$ fraction even at levels considerably greater than those present in the $\gamma\epsilon$-rich fraction. Although ϵ is a potent inhibitor of purified EC-F_1, the failure of ϵ to restore activity to the $\alpha\beta$ fraction was not due to an inhibition of reconstituted enzyme since the added ϵ did not prevent reconstitution of the $\alpha\beta$ fraction by the $\gamma\epsilon$-rich fraction. Somewhat less activity was restored in the presence of the highest level of ϵ but this probably results from the partial inhibition of the reconstituted enzyme rather than inhibition of the reconstitution process. The ϵ added before reconstituting the ATPase was only slightly inhibitory since the inhibition by ϵ is reversed by the dilution of the enzyme prior to its assay (unpublished results). If ϵ was added after diluting the enzyme, the reconstituted ATPase was as sensitive to the inhibitory subunit as the enzyme before cold inactivation. Although the results strongly suggest that γ is required for assembling the catalytic unit from dissociated subunits, we do not yet know

if γ is sufficient or if it is required along with ϵ for the reconstitution of the enzyme after cold inactivation.

Since we have used purified EC-F_1 missing the δ subunit for the studies reported here, δ clearly is not necessary for the restoration of ATPase activity after cold inactivation. We wanted to find out, however, if δ by itself or in combination with the ϵ subunit could substitute for γ in the restoration of catalytic activity to the $\alpha\beta$ subunits. Therefore, purified δ (Smith and Sternweis, 1977) and a fraction containing chiefly the δ and ϵ subunits (Smith and Sternweis, 1975), were combined with the $\alpha\beta$ fraction. δ alone, like ϵ, did not restore any activity to the $\alpha\beta$ fraction, nor did the fraction containing both δ and ϵ. Moreover, neither δ, nor δ plus ϵ, inhibited reconstitution of the $\alpha\beta$ fraction by the $\gamma\epsilon$-rich fraction. These results indicate that δ and ϵ, either alone or in combination, are unable to restore catalytic activity to the $\alpha\beta$ fraction.

To find out if the excess γ was active by itself in the assembly of the enzyme, we reactivated all the α and β in the $\gamma\epsilon$-rich fraction before mixing it with the $\alpha\beta$ fraction. If the excess γ were acting independently of the α and β in the $\gamma\epsilon$-rich fraction, then after first assembling the α and β in the $\gamma\epsilon$-rich fraction, the remaining γ should reactivate additional α and β. This prediction was confirmed since after first reconstituting the α and β in the $\gamma\epsilon$-rich fraction by dialysis against MgATP, this fraction retained 76% of its capacity to reactive added α and β. The slight loss of reconstituting activity probably results from the inactivation of γ during the first dialysis.

The prior dialysis of the $\alpha\beta$ fraction against MgATP, before mixing with the γ containing fraction, which of course does not restore any ATPase activity, also decreased by about 25% the amount of ATPase which was reconstituted when the two fractions were combined without any prior dialysis. Also, about 50% less activity was reconstituted when both fractions were dialyzed separately before being combined.

Sensitivity of EC-F_1 to the Inhibitory ϵ Subunit. Previously we showed that protease digested enzyme containing only the two larger subunits ($\alpha\beta$-ATPase) was insensitive to the inhibitory ϵ subunit in contrast to enzyme containing γ and some endogenous ϵ in addition to α and β (Smith et al., 1975; Smith and Sternweis, 1977). This result suggested that γ of EC-F_1 was essential for inhibition by ϵ and similar results were previously reported for the chloroplast F_1 (Nelson et al., 1974). More substantial evidence that purified γ is essential for ϵ to inhibit the ATPase would be to show that γ restores ϵ sensitivity to the $\alpha\beta$-ATPase. The results in Table I show that the $\alpha\beta$-ATPase is completely insensitive to the inhibitory subunit unless the γ containing fraction is added which makes it completely sensitive to ϵ as would be expected if γ were essential for ϵ inhibition.

Before the experiment the $\alpha\beta$-ATPase was about 50% inactivated by exposure to low temperature. Thus the twofold activation of the $\alpha\beta$-ATPase after dialysis against MgATP at room temperature with added γ subunit probably results from the γ-dependent reconstitution of the dissociated mixture of α and β subunits (Table I).

Viscosity of the Subunit Fractions. After dialyzing the $\alpha\beta$ fraction against MgATP at room temperature, it became remarkably viscous, but remained devoid of ATPase activity as already mentioned. The intrinsic viscosity of the $\alpha\beta$ fraction was measured in a capillary viscometer (Cannon-Ubbelohde Semi-micro Dilution, Cannon Instrument Co.) and found to be about 10 000 mL/g. By contrast, the native enzyme had an intrinsic viscosity of only about 6 mL/g. The $\gamma\epsilon$-rich fraction did not show the high viscosity of the $\alpha\beta$ fraction, suggesting that the presence of the smaller subunits interferes with the viscosity of the α and β subunits.

TABLE I: Effect of the γ Subunit on the Sensitivity of Trypsin-Treated ECF_1 to Inhibition by the ϵ Subunit.[a]

Additions	ATPase (U) No ϵ	ATPase (U) $+\epsilon$	% inhibition
1. None	6.6	6.6	0
2. $\gamma\epsilon$-rich fraction	14.5[b]	2.9	80

[a] The $\alpha\beta$-ATPase was precipitated four times with ammonium sulfate (65% saturation) and dissolved in SED buffer and frozen. The high salt buffer used to inactivate the four-subunit enzyme was avoided because the reconstitution of ATPase activity was poor when this buffer was used to completely inactivate the $\alpha\beta$ enzyme. The $\alpha\beta$-ATPase (160 μg) was dialyzed overnight at room temperature by itself or with 60 μg of the $\gamma\epsilon$-rich fraction. [b] The 6.0 units of ATPase activity which is due to the reconstitution of the α and β subunits present in the $\gamma\epsilon$-rich fraction was subtracted. The sensitivity to 2.0 μg of the purified ϵ subunit was assayed as previously described (Smith and Sternweis, 1977).

Discussion

Many multimeric enzymes are known to be less stable at 4 °C than at ambient temperature (see Penefsky and Warner, 1965; Jarabak et al., 1966; or Irias et al., 1969, for compilations). The F_1 portion of the proton-translocating ATPase from bacteria, mitochondria, and chloroplasts is cold labile (Pullman et al., 1960; Penefsky et al., 1960; McCarty and Racker, 1966; Abrams and Smith, 1974) and the loss of activity during incubation in the cold is known to be accompanied by dissociation into subunits (Penefsky and Warner, 1965; Lien et al., 1972; Vogel and Steinhart, 1976). Recent studies of the *E. coli* F_1 ATPase by Vogel and Steinhart (1976) have shown that incubation with MgATP restores the activity lost by cold inactivation and this was confirmed here. In the present study and previously (Smith et al., 1976), essentially complete reactivation was achieved by dialyzing the subunits in the presence of both Mg^{2+} and ATP (Figure 2) providing the γ subunit was present. The requirement for γ to restore catalytic activity after dissociating the ATPase into subunits which is reported here was somewhat unexpected since γ does not appear to be a component of the catalytic site per se.

Removal of γ as well as δ and ϵ from EC-F_1 by digestion with trypsin does not decrease the ATPase activity of the enzyme implying that the two larger α and β chains are sufficient for ATPase activity (Nelson et al., 1974; Smith et al., 1975). From these observations it may be inferred that γ is essential for the *assembly* of α and β into the catalytic unit. Once the catalytic unit has formed, γ may be removed leaving an intact catalytic unit composed only of α and β chains. Besides the demonstrated role of γ in the assembly of the α and β chains, γ does appear to have a role in energy transduction by the complete proton-pump ATPase since a specific modification of γ in CF_1 reduces photophosphorylation (McCarty and Fagan, 1973).

The results presented here provide a rudimentary basis for an in vitro assembly pathway of the F_1-ATPase subunits from *E. coli*. Apparently α or β has to combine with γ or γ and ϵ in order to reform the catalytic unit. Vogel and Steinhart (1976) isolated a subfragment of EC-F_1 that contained only the α, γ, and ϵ subunits which was inactive and required addition of the β subunit for reconstitution of ATPase activity. Thus in our experiments it may be that α combines with γ before interacting with β. Once an active catalytic unit has been formed from α, β, and γ, it is probably quite stable since γ does not appear to dissociate from the rest of the molecule. If γ could dissociate from EC-F_1 during assembly, then cold-inactivated EC-F_1 which contains the native proportions of subunits should be able to catalyze the assembly of active enzyme from the α

and β subunits. However, the cold inactivated EC-F_1 containing the native proportion of subunits did not reactivate the $\alpha\beta$ fraction indicating that γ acts in a stoichiometric manner, as opposed to catalytically, during the reassembly of the enzyme (unpublished results). Moreover, the amount of the $\alpha\beta$ fraction which can be reactivated by the $\gamma\epsilon$-rich fraction agrees well with the estimation of the relative excess of γ present.

EC-F_1 from which γ, δ, and ϵ have been removed by digestion with trypsin has full ATPase activity but is no longer sensitive to inhibition by the purified ϵ subunit (Smith et al., 1975, 1977). Since the δ subunit is known not to be required for inhibition by ϵ, it appeared that the removal of γ from EC-F_1 caused it to become insensitive to ϵ. The argument would be considerably strengthened, however, if adding γ back to trypsin-treated EC-F_1 could be shown to restore sensitivity to ϵ. Table I shows that the $\gamma\epsilon$-rich fraction restored full ϵ sensitivity to the EC-F_1 from which γ, δ, and ϵ had been removed by digestion with trypsin. Since the sensitivity to inhibition by ϵ was regained when γ was added back, it may be concluded that the insensitivity of EC-F_1 containing only the α and β subunits is due to the absence of γ and not to a defect in α or β. Experiments with cross-linking reagents indicate that the γ and ϵ chains are adjacent to one another in ECF_1 (Bragg and Hou, 1976) and CF_1 (Baird and Hammes, 1976).

Recently Kagawa (1976) reported that the γ chain of the F_1 from a thermophilic bacterium was required for renaturing hydrolytic activity using subunits isolated in urea. While this is consistent with our conclusion about the essentiality of γ, it is not yet known whether the γ chain of this F_1 is a component of the catalytic site for ATP hydrolysis or if γ is required for the assembly of α and β chains as in *E. coli*. Also an inhibitory subunit has not been demonstrated in the thermophile.

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