Vol. 82, No. 2, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 30, 1978 Pages 596-602

IDENTIFICATION OF THE ALTERED SUBUNIT IN THE INACTIVE F_1ATPase OF AN ESCHERICHIA COLI uncA MUTANT

Stanley D. Dunn

Section of Biochemistry, Molecular and Cell Biology
Cornell University, Ithaca, New York 14853

Received April 6,1978

SUMMARY: ATPase activity was restored to the inactive coupling factor, F_1ATPase, of Escherichia coli strain AN120 (uncA401) by reconstitution of the dissociated complex with an excess of wild-type α subunit. Large excesses of α gave the highest levels of activity. The other subunits which are required for the reconstitution of ATPase activity, β and γ, did not complement the mutant enzyme. These results indicate that the α polypeptide of the AN120 ATPase is defective.

The coupling factor, F_1ATPase, catalyzes the synthesis and hydrolysis of ATP in reactions coupled to the electrochemical proton gradient (for reviews, see 1-3). Study of the F_1ATPase (ECF_1) of Escherichia coli, which is similar in structure to those of mitochondria and chloroplasts, has the distinct advantage that E. coli is amenable to genetic analysis.

In 1971, Butlin and coworkers (4) described a mutant strain, AN120 (uncA401), which is incapable of oxidative phosphorylation and lacks ATPase activity. Since then, many other ATPase mutants have been isolated (5-8). Bragg and Hou (9) purified the inactive ECF_1 of strain AN120 and found that it was similar to the parental complex in subunit composition and bound nucleotide content. However, the identity of the altered polypeptide has not been reported previously.

Methods for the cold-dissociation of ECF_1 and the reconstitution of ATPase activity from the dissociated subunits have been described (10,11). In addition, a method for the isolation of pure active subunits has been described by Futai (12), and an alternate procedure has been developed (S.D. Dunn, unpublished results). The three major subunits, α, β and γ, are all required for the reconstitution of ATPase activity (12). These developments permit the identification of the altered subunit in a mutant ATPase by

0006-291X/78/0822-0596$01.00/0

determining which pure wild-type subunit will complement the mutant ATPase to produce activity. α is shown in this paper to be the altered polypeptide in the inactive ECF_1 of strain AN120.

MATERIALS AND METHODS

Preparation of active subunits. Pure ECF_1 was prepared from E. coli K12(λ) by the method of Futai et al. (13) with the modification of Smith and Sternweis (14). The complex was dissociated by the procedure of Futai (12) with the exception that sodium salts were used in place of potassium salts, and the $NaNO_3$ concentration was 250 mM. Active subunits were isolated by chromatography on hydroxyapatite (Bio-Gel HTP, Bio-Rad Laboratories) and DEAE-Sepharose (Pharmacia Co.); the procedure will be published in detail elsewhere.

Preparation of inactive ECF_1. E. coli strain AN120 (uncA401), kindly provided by Dr. Frank Gibson, was grown in the basic salts medium of Tanaka et al. (15) supplemented with 0.5% glucose, 5% (V/V) L broth and the following, in μg/ml; L-arginine, 3.4; L-histidine, 5.0; L-proline, 5.0; thiamine, 0.1; L-isoleucine, 5.0; L-valine, 5.0; L-methionine, 5.0; and casamino acids, 15.0.

The preparation of membranes and extraction of the inactive ATPase were as described for strain K12(λ). The complex was partially purified from the membrane extract by the following procedure, which is a modification of the method of Vogel and Steinhart (10) devised by L. Heppel. The extract (27 ml, 0.63 mg/ml) was cooled to -10° as methanol was added to 20% (V/V). Then 1 M $MgCl_2$ was added to a final concentration of 50 mM and a precipitate developed. After 5 min the suspension was centrifuged at -10° for 5 min at 20,000 x g. The precipitate was extracted with 3.75 ml of buffer containing 1 mM Tris-Cl, pH 8.0, 0.5 mM EDTA, 0.5 mM dithiothreitol and 10% glycerol. After 10 min an equal volume of water was added. Five min later the suspension was centrifuged (30 min at 100,000 x g) and the supernatant fraction contained ECF_1 (7.5 ml, 0.82 mg/ml). The inactive ATPase was dissociated by the same procedure used for K12(λ) ATPase except that the dialysis was omitted.

Reconstitution of ATPase activity. Pure subunits were added to the preparation of dissociated ECF_1 from AN120 and the mixtures were dialyzed overnight at 22-25° C against reconstitution buffer containing 50 mM succinate-Tris, pH 6.0, 5 mM ATP (Sigma, Grade II), 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol and 10% glycerol. ATPase activity was assayed by the colorimetric method of Futai et al. (13); one unit of activity hydrolyzes 1 μmol ATP/min. Protein was assayed by the method of Bradford (16) using bovine serum albumin as a standard. Pure ECF_1 isolated from strain K12(λ) had a specific activity of 110-120 U/mg.

RESULTS

Preparation of ECF_1 from strain AN120. The inactive ECF_1 of strain AN120 behaved similarly to the wild-type complex in its release from membranes by the low ionic strength extraction buffer. From densitometer scans at 590 nm of 10% polyacrylamide gels run in the presence of sodium dodecyl sulfate (17) and stained with Coomassie blue R-250, it was estimated that the major

subunits of ECF_1, α,β and γ, account for 30% of the protein in the crude membrane extract and 60% of the protein in the partially purified preparation. Thus the methanol-$MgCl_2$ step gave a 2-fold purification with 73% recovery. The mutant ECF_1 preparation contained essentially no ATPase activity (<0.1 U/mg of protein).

Restoration of ATPase activity by added α subunit. The ability of the subunits prepared from strain K12(λ) to restore ATPase activity to the cold-dissociated mutant ECF_1 was tested at several subunit concentrations (Table I). Pure α subunit restored significant activity at all concentrations tested while β and γ were ineffective. This complementation indicates that the α subunit of the mutant complex is defective. Dissociation of the mutant complex was essential for the complementation by α. Repeated freezing and thawing of the mutant preparation did not alter the extent of reconstitution.

The purified subunits were essentially inactive either individually or in pairs. Enzyme reconstituted from a mixture of the three wild-type subunits had a specific activity of 76 U/mg of protein.

Effect of α concentration on restoration of ATPase activity. The level of ATPase activity restored to the AN120 ECF_1 rose with increasing amounts of wild-type α (Fig. 1). It is estimated that 39 μg of AN120 protein contained about 11 μg of α. Therefore a large excess of added α is necessary for maximal reconstitution, although levels much lower than equivalence did produce significant activity. This suggests that the defective α subunit of strain AN120 competes effectively with the active subunit for the α sites in the complex.

Effect of purity on reconstitution of ATPase activity. Although pure active ECF_1 which has been cold-dissociated reconstitutes essentially full activity (11), a crude membrane extract of strain K12(λ), with an initial specific activity of 31 U/mg of protein, reconstituted only 7.9 U/mg of protein. Other proteins in the crude extract apparently interfere with reconstitution.

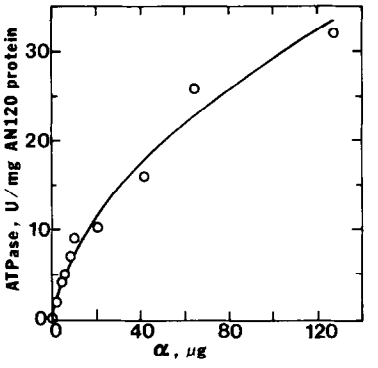

Fig. 1. Effect of α concentration on complementation of AN120 ECF_1. Various amounts of α were mixed with 39 μg of cold-dissociated, partially purified AN120 ECF_1 in a final volume of 0.12 ml. Reconstitution and determination of activity were as described for Table I.

A similar effect was observed in the complementation of the mutant AN120 ATPase by active α. A mixture of α (42 μg) and crude membrane extract protein (78 μg, cold-dissociated) reconstituted only 3.1 U/mg of AN120 protein. In contrast, 16 U/mg of AN120 protein was obtained when an amount of the more purified preparation with about the same ATPase content was reconstituted with α (Table I, line 3).

DISCUSSION

The inactive F_1ATPase of strain AN120 (uncA401) was complemented by pure wild-type α subunit, but not by β or γ. As all three subunits are required for the reconstitution of ATPase activity, the β and γ subunits of the mutant are active, and α is defective. Vogel and coworkers (18) have used complementation studies with partially resolved subunits to show that four ATPase mutants produce active β subunit, but the inactive polypeptides were not identified.

The fact that the mutant complex lacks ATPase activity strongly suggests that the α subunit is required for catalytic activity. The low levels of ATPase activity reconstituted from mixtures of the isolated β and γ subunits of the ECF_1 of strain K12(λ) reported here (Table I) and by Futai (12) may

TABLE I

Restoration of ATPase Activity to the Mutant ECF_1 of Strain AN120 by α.

Protein added (μg)				ATPase Activity Reconstituted	
AN120 ECF_1	α	β	γ	U/mg protein (total)	U/mg protein (AN120)
97	-	-	-	<0.1	<0.1
39	8	-	-	5.9	7.2
39	42	-	-	7.7	16
50*	42	-	-	0.1	0.2
39	-	7	-	0.1	0.1
39	-	35	-	0.1	0.3
39	-	-	2	0.1	0.1
39	-	-	6	0.2	0.2
-	42	-	-	<0.1	-
-	-	52	-	<0.1	-
-	-	-	4	<0.1	-
-	25	21	-	0.4	-
-	25	-	4	<0.1	-
-	-	21	4	1.0	-
-	25	21	4	76	-

The pure subunits and the cold-dissociated, partially purified AN120 ECF_1 were mixed in a total volume of 0.12 ml and dialyzed overnight against reconstitution buffer as described in Methods. The ATPase activity and protein concentration were determined. Activity per mg of AN120 protein was calculated from the specific activity obtained and the composition of the protein added initially.

* The AN120 ECF_1 complex was not cold-dissociated.

be due to slight contamination of these subunits by α. The inactive ECF_1 of strain AN120 restores respiration-dependent transhydrogenase activity to membranes depleted of ATPase (9) indicating that the alteration of α does not impair the ability of the complex to decrease the permeability of the membranes to protons.

The highest activities were obtained with a large excess of active α subunit, suggesting that the α of strain AN120 competes with the added active subunit. Although there is no general agreement on the subunit stoichiometry of ECF_1 (3), the evidence indicates that there are more than

one α subunits per molecule of ATPase. The linearity of Fig. 1 at low α concentrations suggests that complex species containing a mixture of the two types of α are partially active; one would expect a sigmoidal curve if inclusion of a single mutant α subunit inactivated an ECF_1 complex. Although the results are not conclusive, this interpretation is consistent with the observation (19) that $\underline{unc}^+/\underline{uncA401}$ partial diploids produce ATPase activity equivalent to that of haploid $\underline{unc}^+$ strains.

Isolation of the subunits of the mutant ECF_1 is currently in progress. Complementation experiments using combinations of subunits from strains K12(λ) and AN120 should provide confirmation of α as the mutant polypeptide and further information on the functional roles of the subunits.

This technique of analyzing mutant enzyme complexes has been used successfully in the study of E. coli RNA polymerase (20). Identification of the altered subunits of E. coli ATPase mutants by this method does not require extensive purification of the mutant enzyme or isolation of its subunits. However, the technique is most applicable to strains with structurally normal ECF_1 complexes.

Acknowledgements. This work was done in the laboratory of Dr. Leon A. Heppel, whom I thank for advice and encouragement. I also thank Ms. Sharon Johnston, Gloria Kulhawy and Chi Chi Lau for technical assistance. This work was supported by Grant No. AM-11789 and Fellowship No. 1F32 GM06492-01 from the National Institutes of Health and Grant No. BMS 75-20287 from the National Science Foundation.

REFERENCES

1. Harold, F.M. (1977) Curr. Top. Bioenerg. 6, 83-149.
2. Haddock, B.A. and Jones, C.W. (1977) Bacteriol. Rev. 41, 47-99.
3. Wilson, D.B. and Smith, J.B. (1978) Bacterial Transport, in press, Marcel Dekker, New York.
4. Butlin, J.D., Cox, G.B. and Gibson, F. (1971) Biochem. J. 124, 75-81.
5. Kanner, B.I. and Gutnick, D.L. (1972) J. Bacteriol. 111, 287-289.
6. Simoni, R.D. and Shallenberger, M.K. (1972) Proc. Nat. Acad. Sci. USA 69, 2663-2667.
7. Schairer, H.U. and Haddock, B.A. (1972) Biochem. Biophys. Res. Commun. 48, 544-551.
8. Rosen, B.P. (1973) J. Bacteriol. 116, 1124-1129.
9. Bragg, P.D. and Hou, C. (1977) Arch. Biochem. Biophys. 178, 486-494.
10. Vogel, G. and Steinhart, R. (1976) Biochemistry 15, 208-216.
11. Larson, R.J. and Smith, J.B. (1977) Biochemistry 16, 4266-4270.
12. Futai, M. (1977) Biochem. Biophys. Res. Commun. 79, 1231-1237.

13. Futai, M., Sternweis, P.C. and Heppel, L.A. (1974) Proc. Nat. Acad. Sci. USA 71, 2725-2729.
14. Smith, J.B. and Sternweis, P.C. (1977) Biochemistry 16, 306-311.
15. Tanaka, S., Lerner, S.A. and Lin, E.C.C. (1967) J. Bacteriol. 93, 642-648.
16. Bradford, M.M. (1976) Analytic. Biochem. 72, 248-254.
17. Laemmli, U.K. (1970) Nature 227, 680-685.
18. Vogel, G., Schairer, H.U. and Steinhart, R. (1976) Int. Cong. Biochem. Abstr., 10th, 340.
19. Gibson, F., Cox, G.B., Downie, J.A. and Radik, J. (1977) Biochem. J. 162, 665-670.
20. Zillig, W., Palm, P. and Heil, A. (1976) RNA Polymerase, pp. 101-125, Cold Spring Harbor Lab., Cold Spring Harbor, NY.