CROSS-LINKING OF MINOR SUBUNITS IN Ca^{2+}, Mg^{2+}-ACTIVATED ATPase OF ESCHERICHIA COLI

P.D. Bragg and C. Hou
Department of Biochemistry
University of British Columbia
Vancouver, B.C., Canada V6T 1W5

Received July 26,1976

SUMMARY

The subunits of the E. coli ATPase molecule were cross-linked with the cleavable disulfide cross-linking agent, dithiobis (succinimidyl propionate). Cross-linking between α and β, α and δ, β and γ, β and ε, and γ and ε subunits was observed. No αα, ββ, βδ, γδ, or δε cross-linked products were found. Although a unique arrangement of all the subunits of the enzyme cannot be proposed from these data, the interactions of the minor subunits are demonstrated for the first time.

INTRODUCTION

The membrane bound Mg^{2+}-activated ATPases of chloroplasts, mitochondria and many bacteria, including E. coli, play an essential role in processes such as photophosphorylation, oxidative phosphorylation, and some ATP-dependent reactions such as active transport (1,2). These functionally similar enzymes are composed of five different types of subunits (α-ε) the molecular weights of which differ somewhat for the enzymes from the different systems. The stoichiometric ratio of these subunits is probably $\alpha_3\beta_3\gamma\delta\varepsilon$ (1-3). The arrangement of the subunits in the ATPase molecule has still to be determined. Electron micrographs of the enzyme from mitochondria and bacteria suggest that six subunits are arranged as a planar hexagon around a central subunit (1,4,5). It has been proposed that the six subunits of the hexagon are the α and β subunits probably arranged alternately (1,3,6). There

---

Abbreviations: DSP, dithiobis(succinimidyl propionate); SDS, sodium dodecyl sulfate.

is little information on the arrangement of the three smallest subunits. In the present paper we have examined the relationship of the smaller subunits to the α and β subunits, and to themselves, by means of cross-linking.

METHODS

The Ca^{2+}, Mg^{2+}-activated ATPase of E. coli ML308-225 was purified for cross-linking studies as previously described (3). The ATPase fractions from the sucrose gradient were transferred into 0.05 M triethanolamine buffer, pH 8.3, containing 10% glycerol by dilution with this buffer followed by reconcentration to 1.5-2.0 mg protein/ml by ultrafiltration with an Amicon XM-100 filter. The enzyme was treated at 23°C with 0.02 ml/ml of DSP (Pierce Chemical Company) (20 mg/ml in dimethylsulfoxide). After 0.5-1.0 min 0.1 ml/ml 4 M Tris-HCl buffer, pH 7.8, was added followed after 1 min by 0.1 ml/ml 10% SDS. The solution was heated at 100°C for 3 min and then submitted to gel electrophoresis.

The ATPase (2 mg protein/ml) was cold-salt denatured by the method of Vogel and Steinhart (7). The enzyme was renatured by their procedure at pH 6.1. The denatured enzyme was also maintained in this state by replacing the renaturation buffer with 0.1 M triethanolamine, pH 8.0. For cross-linking, the native, denatured and renatured enzymes were exchanged into the cross-linking buffer system as above.

SDS-polyacrylamide gel electrophoresis was carried out on 0.75 mm thick gel slabs in a Bio Rad Model 220 electrophoresis apparatus run at 30 mA per slab. The buffer and gel systems of King and Laemmli (8) were used except that the stacking gel was 5% cyanogum (EC Apparatus Corp.). For two-dimensional electrophoresis a 3-4 mm wide strip was cut from the 7.5% acrylamide first-dimension gel and placed horizontally above the stacking gel of a 1.5 mm thick gel slab. The strip was surrounded by 1% agarose in 0.125 M Tris-HCl buffer, pH 6.8, containing 0.1% SDS and 2% β-mercaptoethanol, and run at 40 mA per slab. The 11% acrylamide second-dimension gel was prepared the previous day and stored with the stacking gel overlaid with the above buffer but containing 10% β-mercaptoethanol.

Gels were stained and destained by the procedure of Fairbanks et al. (9).

RESULTS

In a previous paper we had found a single major cross-linked product identified as α linked to β following treatment of the ATPase of E. coli with DSP. Cross-linked products containing the minor subunits were not detected (3). At a lower ratio of DSP to ATPase than was used previously seven bands with molecular weights smaller than the

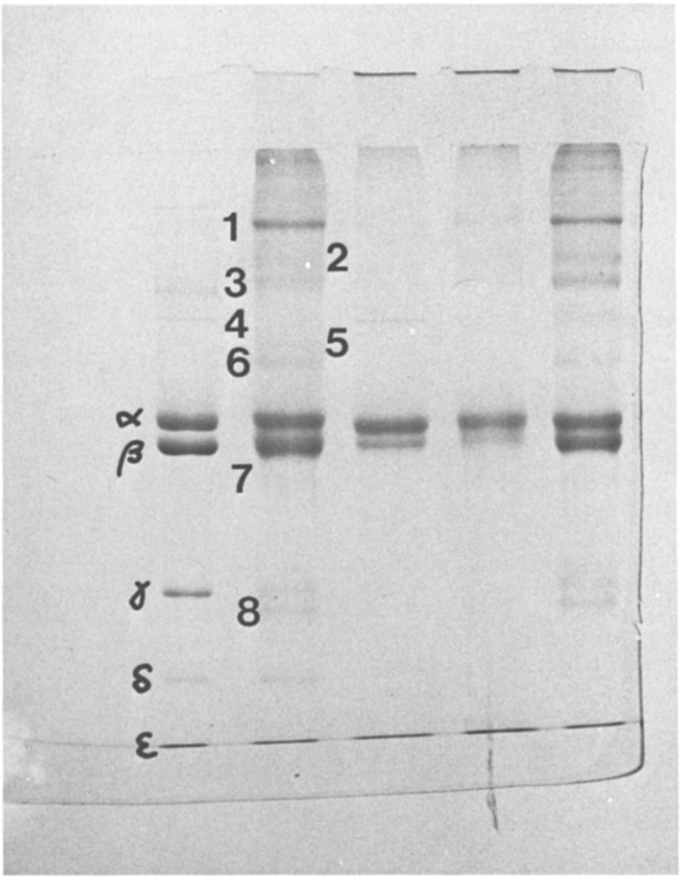

FIGURE 1. SDS-polyacrylamide gel electrophoresis of native, cold-salt denatured, and renatured ATPase following cross-linking with DSP. From the left the channels are untreated native ATPase, cross-linked native ATPase, cross-linked denatured ATPase (one-half level of DSP), cross-linked denatured ATPase, and cross-linked renatured ATPase. 0.02 ml DSP (40 mg/ml) was added per ml enzyme solution. Bands 1-8 indicate cross-linked products. The running gel contained 9% acrylamide.

αβ band were found on polyacrylamide gel electrophoresis in SDS (Fig. 1, bands 2-8). Bands 2-6 could be resolved into double bands under some conditions of electrophoresis although bands 1 and 8 always remained as single, discrete bands.

That the observed cross-linking products were related to the native structure of the ATPase was shown as follows. Vogel and Steinhart (7) found that the ATPase of E. coli lost its activity on

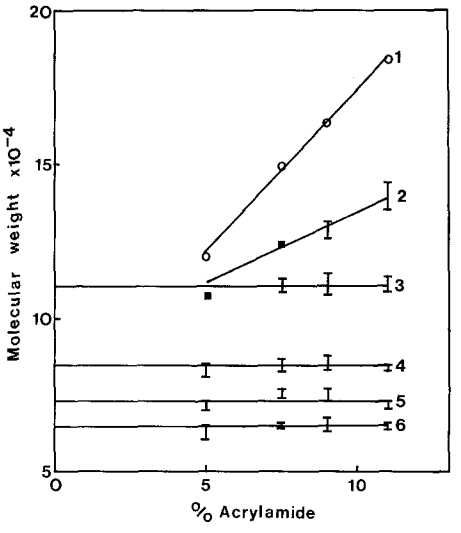

FIGURE 2. Dependence of the molecular weights of bands 1-8 on the concentration of acrylamide in the running gel. The molecular weights were determined at each concentration of acrylamide by comparison with the rate of migration of β-galactosidase, phosphorylase a, bovine serum albumin, and the β and β^1 subunits of RNA polymerase. Where multiple components were observed in a particular band the molecular weights of the lowest and highest molecular weight components of the band are joined by a bar.

freezing in salt solutions with dissociation of the β subunits from the molecule. Enzyme activity was recovered when the dissociated enzyme was incubated at pH 5-6 at temperatures above 18°C. We have confirmed these observations. As shown in Fig. 1, channels 3 and 4, the products formed on cross-linking the cold-denatured enzyme were different from those given by the native enzyme (channel 2). The previous pattern of cross-linking was recovered on renaturation (channel 5).

The molecular weights of bands 1-6 were determined by SDS gel electrophoresis. Those of bands 1 and 2 were strongly dependent on the concentration of polyacrylamide such that a reliable estimate could not be made (Fig. 2). The value of 120,000 for the molecular weight of band 1 obtained with the 5% gel is about 10% higher than the theo-

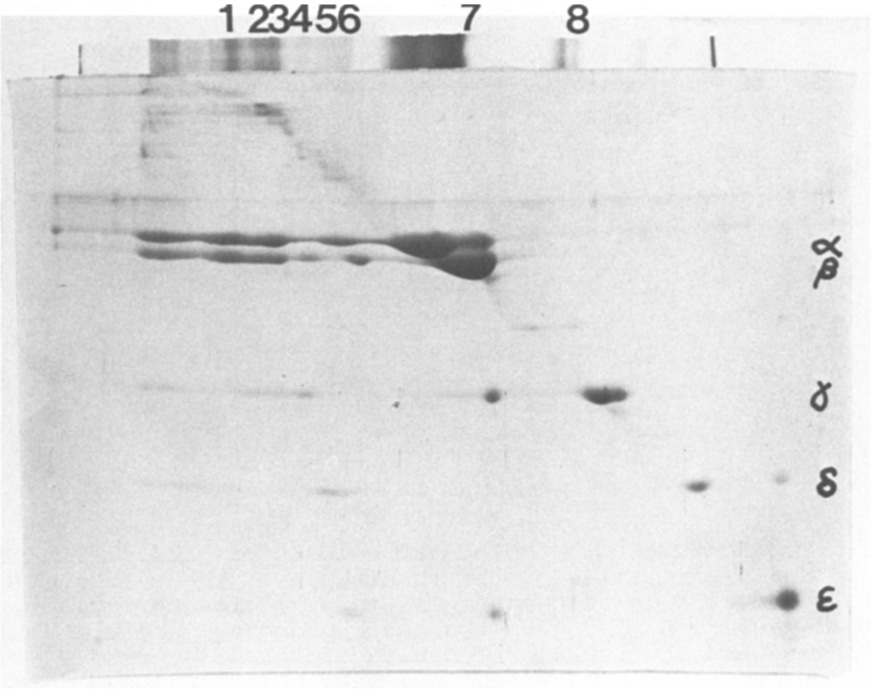

FIGURE 3. Two-dimensional SDS-polyacrylamide gel electrophoresis of DSP-treated ATPase. A stained duplicate strip of the first-dimension gel is placed over the second dimension gel for identification of the cross-linked products (bands 1-8). The direction of migration is from left to right in the first dimension, and from top to bottom in the second dimension.

retical value of 108,600 for an αβ product. The molecular weights of bands 3 to 6 were 111,000, 85,000, 73,000 and 65,000, respectively.

The identity of the cross-linked products in bands 1 to 8 was examined by two-dimensional SDS-polyacrylamide gel electrophoresis. The second dimension was run in the presence of β-mercaptoethanol to cleave the disulfide bond of the cross-linker. Under these conditions band 8 gave the γ subunit only (Fig. 3). Since band 8 migrated more rapidly than the native γ subunit it probably consists of the γ polypeptide in which cross-linking has occurred between lysine residues on the same chain. This molecule would not unfold as fully in SDS as

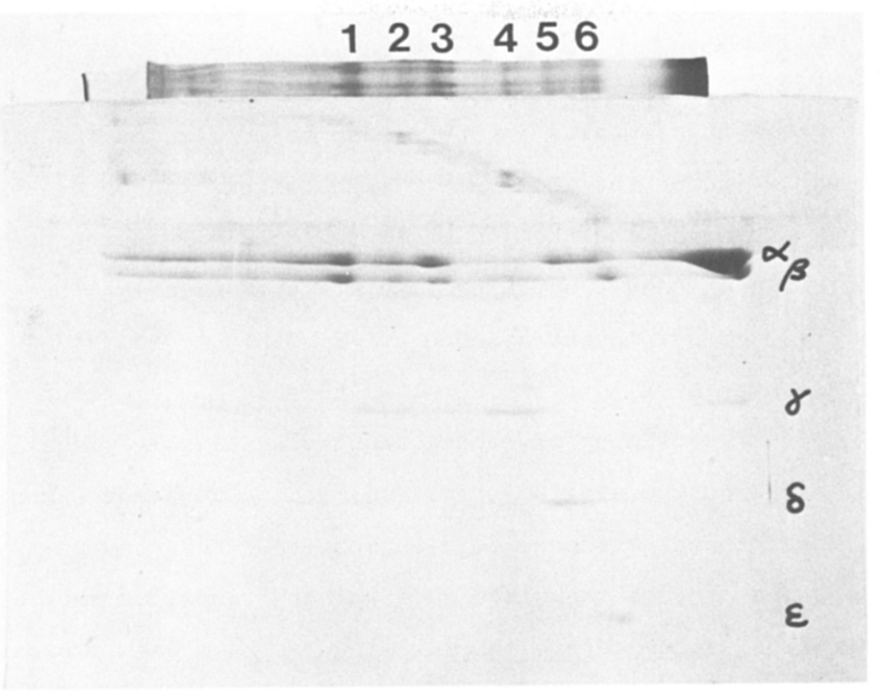

FIGURE 4. Two-dimensional SDS-polyacrylamide gel electrophoresis of DSP-treated ATPase. A stained duplicate strip of the first-dimension gel is placed over the second-dimension gel for comparison. The direction of migration is from left to right in the first dimension, and from top to bottom in the second dimension.

the native polypeptide and so would migrate more rapidly on electrophoresis. Band 7 was composed of both γ and ε subunits (Fig. 3). Its molecular weight of about 45,000 is consistent with a $\gamma\varepsilon$ structure.

The molecular weights and cleavage products of bands 4,5, and 6 are consistent with $\beta\gamma$, $\alpha\delta$, and $\beta\varepsilon$ cross-linked products (Fig. 4). The double bands observed may be due to cross-linking within one or both of the polypeptide chains. Thus, two $\alpha\delta$ cross-linked products are seen. Certain impurities present in the ATPase preparation migrate in this region and may account for the multiple nature of band 4. We have not rigorously identified the nature of bands 2 and 3 although $\alpha\gamma$ and $\beta\gamma$ products may be present in this region of the gel. Band 1 is $\alpha\beta$.

DISCUSSION

The present results do not permit the assignment of a unique arrangement to the subunits in the ATPase molecule. On the basis of the present evidence the α subunit must be within 12Å of β, δ, and possibly γ subunits, and the β subunit the same distance from γ and ε subunits. The γ and ε subunits also are sufficiently close to be cross-linked. The apparent absence of αα, ββ, βδ, γδ, and δε cross-linked products is of interest although this is not rigorous evidence for the lack of proximity of these subunits. Suitably place lysine residues might not be available for reaction on adjacent subunits. However, the present evidence does suggest that the α subunits and the β subunits are not organized as distinct groups, or that all of the minor (γ-ε) subunits are in proximity, as have been proposed in certain models of the ATPase molecule (6,7).

ACKNOWLEDGMENT

This work was supported by a grant from the Medical Research Council of Canada.

REFERENCES

1. Bronk, J.R. (1974) Membrane Adenosine Triphosphatases and Transport Processes, Biochemical Society, London.
2. Pedersen, P.L. (1975) J. Bioenergetics 6, 243-275.
3. Bragg, P.D., and Hou, C. (1975) Arch. Biochem. Biophys. 167, 311-321.
4. Schatz, G., Penefsky, H.S., and Racker, E. (1967) J. Biol. Chem. 242, 2552-2560.
5. Munoz, E., Freer, J.H., Ellar, D.J., and Salton, M.R.J. (1968) Biochim. Biophys. Acta 150, 531-533.
6. Kozlov, I.A., and Mikelsaar, H.N. (1974) Fed. Eur. Biochem. Soc. Lett. 43, 212-214.
7. Vogel, G., and Steinhart, R. (1976) Biochemistry 15, 208-216.
8. King, J., and Laemmli, U.K. (1971) J. Mol. Biol. 62, 465-477.
9. Fairbanks, G., Steck, T.L., and Wallach, D.F.H. (1971) Biochemistry 10, 2606-2617.