Biochimica et Biophysica Acta 851 (1986) 385–394
Elsevier

BBA 42125

Effect of disulfide cross-linking between α and δ subunits on the properties of the F_1 adenosine triphosphatase of *Escherichia coli*

Philip D. Bragg and Cynthia Hou

Department of Biochemistry, The University of British Columbia, Vancouver, British Columbia, V6T 1W5 (Canada)

(Received 12 May 1986)

Key words: F_1-ATPase; ATPase; Subunit crosslinking; Disulfide bridge; (*E. coli*)

Under very mild oxidizing conditions the δ subunit of the F_1-ATPase of *Escherichia coli* can be crosslinked by a disulfide linkage to one of the α subunits of the enzyme. The cross-linked ATPase resembles the native enzyme in the following properties: (1) specific activity; (2) activation by lauryldimethylamine *N*-oxide (LDAO); (3) binding of aurovertin D and ADP; (4) cross-linking products with 3,3′-dithiobis(succinimidyl propionate); (5) binding to ATPase-stripped everted membrane vesicles and the *N,N′*-dicyclohexylcarbodiimide sensitivity of the rebound enzyme. However, the rebound crosslinked ATPase differed from the native enzyme in lacking the ability to restore NADH oxidation – and ATP hydrolysis-dependent quenching of the fluorescence of quinacrine to ATPase-stripped membrane vesicles. It is proposed that the δ subunit is involved in the proton pathway of the ATPase, and that this pathway is affected in the αδ-cross-linked enzyme. The mechanism for activation of the ATPase by LDAO was examined. Evidence against the proposal of Lötscher, H.-R., De Jong, C. and Capaldi, R.A. (Biochemistry (1984) 23, 4140–4143) that activation involves displacement of the ε subunit from an active site on a β subunit was obtained.

Introduction

The ATP synthase (ATPase) of *Escherichia coli* has a central role in the bioenergetics of this organism being involved both in ATP synthesis by oxidative phosphorylation and in ATP hydrolysis to energize various energy-dependent processes associated with the cell membrane. The F_1 portion of the enzyme is composed of five subunits (α–ε) in a stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon$. It has been studied extensively (for recent reviews, see Refs. 1–4). The function of the different subunits of ECF_1 are now becoming clear [1–5]. The active sites of the enzyme are probably located on the β subunits, possibly at interfaces with α subunits [2,6,7]. The α subunits have a binding site for ADP or ATP [8] and it is likely that the α subunits carry the tightly bound, non-catalytic adenine nucleotides which are found in ECF_1 as isolated [9–12]. The γ subunit has a role in organizing the structure of F_1, and the δ and ε subunits in binding the $\alpha_3\beta_3\gamma$ substructure to F_0 in the membrane [5,13–15].

Net ATP synthesis and hydrolysis involves cooperative interactions between active sites on ECF_1 [16]. A mutation which changes a single amino acid in the α subunit [17] can cause inactivation of these reactions by affecting cooperative interactions between α and β subunits [6,18]. We were interested to see if altering the interactions be-

Abbreviations: ECF_1, F_1-ATPase protein of ATP synthase complex of *Escherichia coli*; ECF_0, membrane protein of the ATP synthase complex of *E. coli*; DCCD, *N,N′*-dicyclohexylcarbodiimide; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; LDAO, lauryldimethylamine *N*-oxide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Correspondence address: Department of Biochemistry, The University of British Columbia, 2146 Health Sciences Mall, Vancouver, British Columbia, Canada, V6T 1W5.

tween other subunits of ECF, would affect its enzymatic activities. As described in this paper, we have developed a mild oxidative procedure which causes the formation of a disulfide bridge between sulfhydryl groups on the α and δ subunits of ECF_1. The αδ-cross-linked enzyme has normal catalytic activities, can bind to ECF_1-depleted membranes, but is unable to set up or maintain a transmembrane pH gradient. The significance of these findings will be discussed with respect to the pathway of protons to ECF_1 and the mechanism of the enzyme.

Materials and Methods

Preparation of F_1 ATPase

E. coli strains KY7485 and LE392pRPG54 were used as sources of the ATPase. Strain KY7485 was heat-induced during growth as described by Foster and Fillingame [19]. Strain LE392pRPG54 was grown in the presence of 25 μg/ml chloramphenicol [20]. The F_1 ATPase was prepared as described previously [21]. The F_1 ATPase was released from the membranes by the low ionic strength dialysis procedure described before [22]. The resulting ATPase-stripped everted membrane vesicles from strain ML308-225 were washed by resedimentation from dialysis buffer and suspended in 50 mM Tris-HCl buffer, (pH 7.5), containing 10 mM $MgCl_2$ and 5.5% (w/v) sucrose for use in F_1 binding experiments.

Preparation of αδ-cross-linked ECF_1

ECF_1 from the final step of purification on a sucrose gradient was present at a concentration of 5–7 mg protein/ml of 0.1 M triethanolamine-HCl, 0.5 mM EDTA/1 mM ATP/22.5% (w/v) sucrose buffer at pH 7.5. Samples (up to 100 μl) were freed of the buffer components by the centrifuged column procedure of Penefsky [23]. The column contained 1 ml of Sephadex G-50 equilibrated with 50 mM triethanolamine-HCl buffer (pH 7.5). The enzyme was used generally between 30 and 60 min after emerging in the column effluent.

Binding of ATPase to ATPase-stripped everted membrane vesicles

ATPase (20 μl containing 100–150 μg protein) was incubated at 20°C for 15 min with ATPase-stripped vesicles (2.0 mg protein) in 4 ml 10 mM Hepes-KOH buffer (pH 7.5), containing 300 mM KCl/5 mM $MgCl_2$. After a further 15 min incubation at 0°C the vesicles were sedimented by centrifugation at 50 000 rpm in a Beckman type 65 rotor. The vesicles were resuspended in 0.2 ml 50 mM Tris-HCl, 10 mM $MgCl_2$ buffer (pH 7.5), for assay of ATPase activity. Rebound ATPase activity was corrected for the small residual ATPase activity present in the ATPase-stripped membrane vesicles.

Fluorescence assays

NADH oxidation and ATP hydrolysis-dependent quenching of the fluorescence of quinacrine was measured as follows. Fluorescence was excited at 430 nm and the emission was measured at 505 nm in a Turner model 420 spectrofluorometer connected to a strip chart recorder. The reaction mixture in a final volume of 2.0 ml contained ATPase-stripped membrane vesicles (1.0 mg protein), ATPase (amounts present as indicated in the figures) and 3.3 μM quinacrine in a buffer of 10 mM Hepes-KOH/300 mM KCl/5 mM $MgCl_2$ (pH 7.5). The ATPase and vesicles were incubated in the buffer for 5 min at 20°C before addition of the quinacrine. Quenching was initiated by adding 10 μl 200 mM NADH. The steady-state level of quenching decreased rapidly on exhaustion of dissolved oxygen in the cuvette to the fluorescence level observed prior to the addition of NADH. Fluorescence quenching energized by the hydrolysis of ATP was then measured by adding 10 μl 200 mM ATP.

The fluorescence of aurovertin D in the presence of F_1 ATPase was measured at 20°C in 2.0 ml of a buffer at pH 7.4 which contained ATPase, 10 mM Tris-HCl, 0.25 M sucrose, 0.5 mM EDTA and 1.19 μM aurovertin D. Fluorescence was excited by light at 365 nm and emission was measured at 470 nm. Changes in fluorescence were measured following sequential additions of 10 μl 40 mM ADP/100 μl 40 mM ATP/10 μl M $MgCl_2$, as described in Ref. 6.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Samples were treated with *N*-ethylmaleimide prior to dissolving in SDS as described in Ref. 21.

2-mercaptoethanol was omitted from all sample buffers, unless indicated otherwise. SDS-polyacrylamide gel electrophoresis as performed on 7.5 or 9% (w/v) polyacrylamide gel slabs (0.75 mm thick) in Tris buffer (pH 9.2), using the Laemmli system [24]. Two-dimensional SDS gel electrophoresis involving the cleavage of a disulfide bridge with 2-mercaptoethanol prior to running the second dimension was carried out as before (Ref. 21).

Cross-linking with 3,3′-dithiobis(succinimidyl propionate) and analysis of the cross-linked products followed the procedures described in Ref. 21.

Other procedures

ATPase activity was measured at 37°C in the presence of 2.5 mM $MgCl_2$ as described previously [25].

Protein was determined by the methods of Bradford [26] (for F_1-ATPase) or Lowry et al. [27] (for vesicles) with bovine serum albumin as a standard.

Treatment of ECF_1 with cupric 1,10-phenanthrolinate was carried out as described in Ref. 22. Crosslinking of ECF_1 with EDC followed the procedure of Lötscher et al. [28].

Results

Formation of αδ-cross-linked ECF_1

The final step in the purification of native ECF_1 involves sucrose density gradient centrifugation in the presence of EDTA, ATP and dithiothreitol. Purification of the ECF_1 from strains KY7485 or LE392pRPG54 by sucrose density gradient centrifugation in the absence of these substances yielded a peak of enzyme in which a portion of the ATPase towards the bottom of the gradient contained α subunit crosslinked by a disulfide bridge to the δ subunit. The enzyme towards the top of the peak also was capable of yielding αδ-cross-linked ECF_1 on treatment with cupric 1,10-phenanthrolinate (Fig. 1, left panel). ECF_1 from strain ML308-225 showed little tendency to form αδ cross-links under these conditions. The cross-linking within ECF_1 had not been observed previously, since samples for SDS-polyacrylamide gel electrophoresis were usually treated with 2-mercaptoethanol. This would have cleaved the disulfide bridge between the sulfhydryl groups on the α and δ subunits.

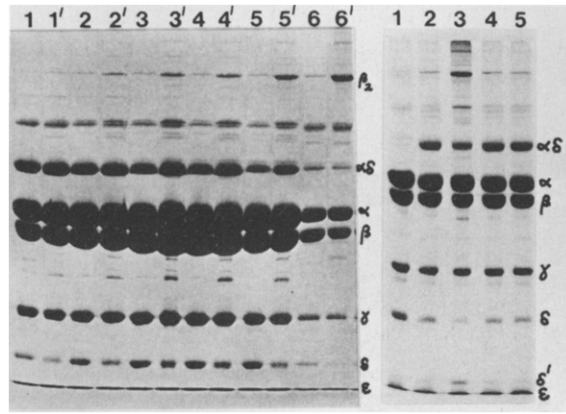

Fig. 1. Conditions leading to the formation of αδ-cross-linked ECF_1. Left panel: SDS-polyacrylamide gel electrophoresis of samples through the ATPase activity peak from the separation of ECF_1 on a sucrose gradient in the absence of dithiothreitol, EDTA and ATP. Samples were prepared for electrophoresis without (lanes 1–6) or with (lanes 1′–6′) a prior treatment with cupric 1,10-phenanthrolinate. Right panel: SDS-polyacrylamide gel electrophoresis of samples from centrifuged column of Sephadex G-50. (1) Native enzyme; (2) enzyme from the Sephadex G-50 column which had been equilibrated with 50 mM triethanolamine buffer (pH 7.5); (3) enzyme from (2) treated with cupric 1,10-phenanthrolinate; (4) enzyme from (2) incubated with 12.5 mM ATP and 6.25 $MgCl_2$ for 30 min at 20°C; (5) enzyme from (4) treated with cupric 1,10-phenanthrolinate. The positions of migration of the α–ε subunits of ECF_1, αδ and β_2 crosslinked dimers, and internally cross-lined δ subunit (δ′) are indicated.

The identification of the cross-linked subunits was confirmed by two-dimensional SDS-polyacrylamide gel electrophoresis in which the first dimension was run in the absence of 2-mercaptoethanol. The second dimension was run after reductive cleavage of the disulfide bond with this thiol. The α and δ subunits released by the cleavage ran as spots off the diagonal containing the non-cross-linked subunits (Fig. 2).

The αδ-cross-linked ECF_1 was prepared more conveniently from the native enzyme purified by sucrose gradient centrifugation by removing the ATP, EDTA and DTT by centrifuging samples of the enzyme through a small column of Sephadex G-50 which was equilibrated with 50 mM triethanolamine-HCl buffer (pH 7.5) [23] (Fig. 1, right panel). As seen in lane 2, passage through Sephadex G-50 resulted in the formation of a

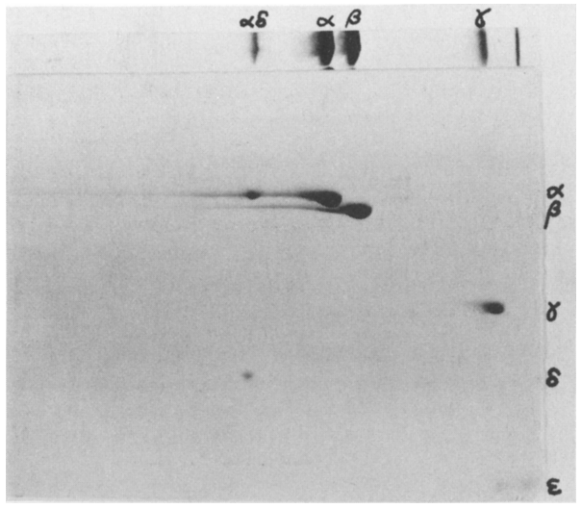

Fig. 2. Two-dimensional SDS-polyacrylamide gel electrophoresis of αδ-crosslinked ECF_1. The direction of migration is from left to right in the first dimension and from top to bottom in the second dimension. A stained duplicate strip of the first-dimension gel is placed at the top of the two-dimensional gel for identification of cross-linked subunits. The subunits arising from cleavage of the disulfide linkage between the cross-linked subunits with 2-mercaptoethanol prior to running the second dimension gel are seen as off-diagonal spots migrating vertically below the αδ band. The position of the α–ε subunits of ECF_1 are indicated.

disulfide cross-link between α and δ subunits, and a decrease in the amount of uncross-linked δ subunit. Treatment of the enzyme from Sephadex G-50 with cupric 1,10-phenanthrolinate did not increase the amount of αδ cross-linking, but did cause the generation of internally cross-linked δ (δ′) and generated a small amount of disulfide-linked β_2 (lane 3). The formation of β_2 and internally crosslinked δ by cupric 1,10-phenanthrolinate was prevented by incubating the enzyme from the column with 12.5 mM ATP/6.25 mM $MgCl_2$ for 30 min at 20°C prior to treatment with this reagent.

Passage of the native ECF_1 through Sephadex G-50 equilibrated with 50 mM triethanolamine-HCl (pH 7.5) containing 0.5 mM EDTA did not lead to the formation of αδ cross-links.

The αδ-cross-linked ECF_1 generated by the Sephadex G-50 procedure sedimented during sucrose gradient centrifugation slightly more rapidly than the native enzyme.

Effect of lauryldimethylamine N-oxide (LDAO) on ATPase activity

The hydrolytic activity of native ECF_1 is stimulated by detergent (117%, 229%, 169%, 127%, 167% and 153% by 0.5% *N*-lauroylsarcosinate, octylglucoside, sodium cholate, sodium deoxycholate, Triton X-100 and Zwittergent 3-12, respectively). Lötscher et al. [28] have reported that the detergent LDAO stimulated the ATPase activity of ECF_1 and proposed that LDAO causes the ε subunit, which has an inhibitory function in ECF_1 [5,15], to be displaced from an active site on a β subunit of the enzyme. The hypothesis was based on the evidence that LDAO stimulated ATPase activity and prevented crosslinking of β to ε by EDC.

LDAO (0.5%) stimulated the ATPase activity of native ECF_1 259% from a specific activity of

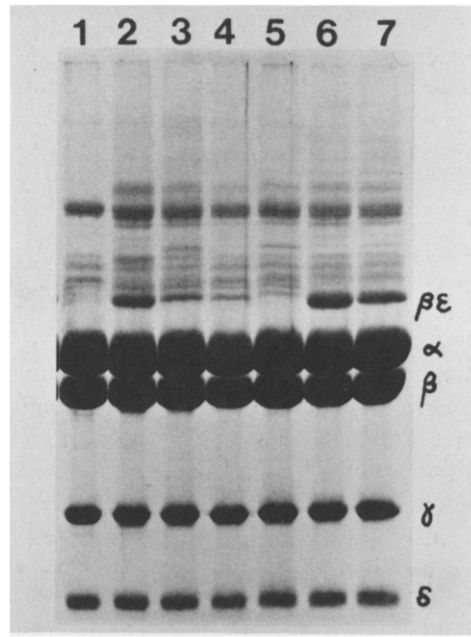

Fig. 3. Effect of LDAO and adenine nucleotides on the formation by EDC of a βε cross-link in ECF_1. Samples prepared as described below are submitted to SDS-polyacrylamide gel electrophoresis. The samples were treated with 2-mercaptoethanol prior to electrophoresis. (1) native enzyme; (2) native enzyme treated with EDC; (3) native enzyme treated with EDC in the presence of 0.5% LDAO; (4) as (3) but with 5 mM $MgCl_2$ present also; (5) as (3) but with 10 mM ATP present also, (6) as (4) but with 10 mM ATP present also; (7) as (4) but with 10 mM ADP present also. The positions of migration of the α–δ subunits of ECF_1 and of the βε cross-linked product are indicated.

18.8 μmol/min per mg protein to 47.7 μmol/min per mg protein. The αδ-cross-linked ECF_1 was stimulated 226% from 25.0 μmol/min per mg protein to 56.4 μmol/min per mg protein. The validity of the mechanism of Lötscher et al. [28] was next investigated, since the presence of the αδ cross-link might be expected to have some effect on the ease with which the ε subunit could be displaced from a β subunit, particularly since it is likely that the δ and ε subunits are relatively close together in the ATPase molecule [15].

Fig. 3 shows, in agreement with Lötscher et al., that the βε crosslink generated by EDC (lane 2) was diminished in the presence of LDAO (lane 3). However, the inhibition of cross-linking by LDAO was prevented by ATP or ADP in the presence of $MgCl_2$ (lanes 6 and 7). ATP or $MgCl_2$ alone could not reverse the inhibition by detergent (lanes 4 and 5). Since ATP and $MgCl_2$ were present in the ATP assay at concentrations similar to those which permitted EDC cross-linking of β to ε, it is unlikely that the stimulation by LDAO necessarily involves displacement of ε from β as suggested by Lötscher et al.

Aurovertin fluorescence assay of native and αδ-crosslinked ECF_1

The fluorescence of aurovertin D is negligible in aqueous solution, but increases markedly on binding of aurovertin D to ECF_1 [6]. The increase in fluorescence was used to measure the K_D for the dissociation of aurovertin from the ECF_1 · aurovertin D complexes formed by native and αδ-cross-linked enzymes. Both enzymes gave K_D values in the range of 1.3–1.5 μM (Fig. 4). The fluorescence of the ECF_1 · aurovertin D complex was enhanced by ADP (or ATP) in a similar manner for both native and αδ-cross-linked enzymes. Both enzymes showed a K_D of 3.7 μM for dissociation of ADP from the ADP · ECF_1 · aurovertin D complex (Fig. 4). As observed previously [6], addition of 2 mM ATP resulted in partial quenching, and 5 mM $MgCl_2$ in full quenching, of the fluorescence of the ADP · ECF_1 · aurovertin D complex. Native and αδ-cross-linked enzymes behaved similarly (data not shown).

Reconstitution of proton translocation

Removal of ECF_1 from everted vesicles of *E. coli* results in leakage of protons through ECF_0. Consequently, a reaction, such as the quenching of the fluorescence of the dye quinacrine, which requires the presence of a transmembrane pH gradient cannot occur. Fluorescence quenching can be observed if the leakage of protons is blocked by readdition of ECF_1. Fig. 5 shows that titration of ECF_1-stripped everted membrane vesicles with native ECF_1 resulted in the restoration of NADH oxidation- and ATP hydrolysis-energized proton translocation as shown by the restoration of the quenching of the fluorescence of quinacrine. Re-

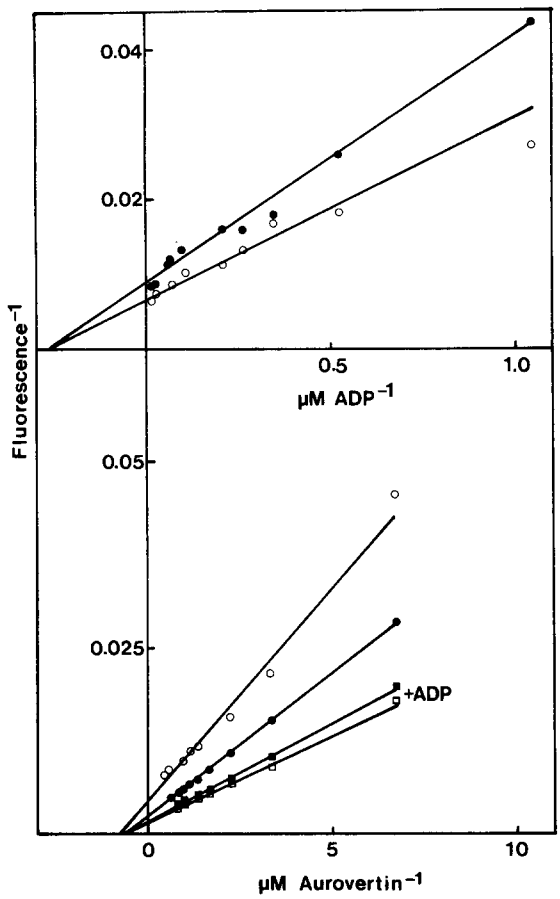

Fig. 4. Double-reciprocal plots of the fluorescence enhancement of aurovertin D with ECF_1 from a Sephadex G-50 centrifuged column equilibrated with triethanolamine buffer in the presence (solid points) or absence (open points) of EDTA. The fluorescence was measured in the presence of increasing concentrations of aurovertin D (bottom panel) or ADP as described in Materials and Methods. In the experiment shown in the bottom panel, each pair of samples was run with and without 0.2 mM ADP. The concentration of protein was 0.5 mg/ml.

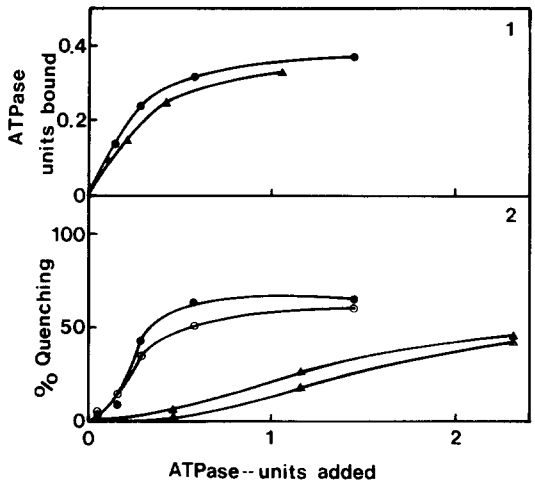

Fig. 5. Rebinding of native (circles) and αδ-cross-linked ECF_1 (triangles) to stripped membrane vesicles (1) and the reconstitution of the fluorescence quenching of quinacrine (2) energized by NADH oxidation (open points) or ATP hydrolysis (solid points). Quenching is expressed as the percentage of the quenching observed before energization. ATPase units are expressed as μmol ATP hydrolyzed/min. The amount of membrane vesicle protein was 0.5 mg/ml reaction mixture. The αδ-crosslinked ECF_1 was prepared by the Sephadex G-50 method.

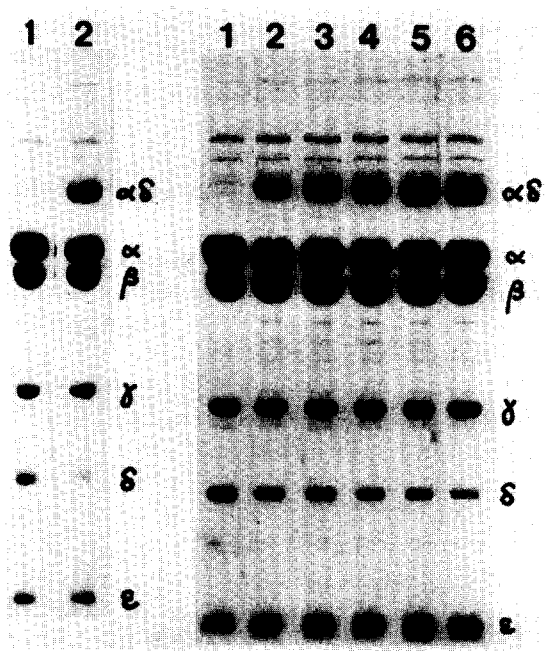

Fig. 6. SDS-polyacrylamide gel electrophoresis of samples used in the reconstitution experiment (left panel) and time-course of the formation of αδ-cross-linked ECF_1 (right panel). Left panel: (1) native ECF_1, used in Fig. 5; (2) αδ-cross-linked ECF_1 used in Fig. 5. Right panel: (1) native ECF_1; (2–6) samples of ECF_1 processed for electrophoresis 3, 6.5, 16, 31 and 60 min, respectively, after emergence of the enzyme from the Sephadex G-50 column. The positions of migration of the α–ε subunits of ECF_1 and αδ-cross-linked subunits are indicated.

storation of quenching was accompanied by the binding of ECF_1 to the stripped vesicles. The αδ-cross-linked ECF_1 bound to the stripped vesicles to an extent similar to that of the native ECF_1. However, fluorescence quenching was not restored to the extent shown by the native enzyme (Fig. 5). The partial restoration which was seen was probably due to the presence of a small amount (20–25%) native ECF_1 in the preparations of αδ-cross-linked ECF_1 (Fig. 6, left panel).

For technical reasons the binding and fluorescence activity were not tested before 30 min had elapsed since the emergence of the sample from the Sephadex G-50 column. Fig. 6 shows the results of an experiment to examine the properties of samples of the enzyme at earlier time periods. In this experiment three types of preparation were examined: ECF_1 which had passed through the column in (a) 50 mM triethanolamine buffer (pH 7.5) containing 0.5 mM EDTA, (b) triethanolamine buffer only, (c) triethanolamine buffer only but supplemented on emergence from the column with 10 mM ATP and 5 mM $MgCl_2$. In the experiments, zero time was the time at which the enzyme was collected from the column. The enzyme was kept at 20°C and at intervals samples were removed for assay of ATPase activity or were added to the stripped membrane vesicle preparations for a 5 min preincubation prior to assaying for ATP-energized quenching of the fluorescence of quinacrine. Other samples were removed, added to stripped membrane vesicles, preincubated at 20°C for 15 min, and then stored at 0°C until all the samples had been collected. The ATPase rebound to the membranes was then sedimented by ultracentrifugation. A further set of samples, removed at intervals, was treated with *N*-ethylmaleimide and prepared for SDS-polyacrylamide gel electrophoresis.

As seen in Fig. 7 (upper panel) there was a rapid loss of the ability of the ATPase which had passed through the column in triethanolamine buffer only to reconstitute ATP-energized fluores-

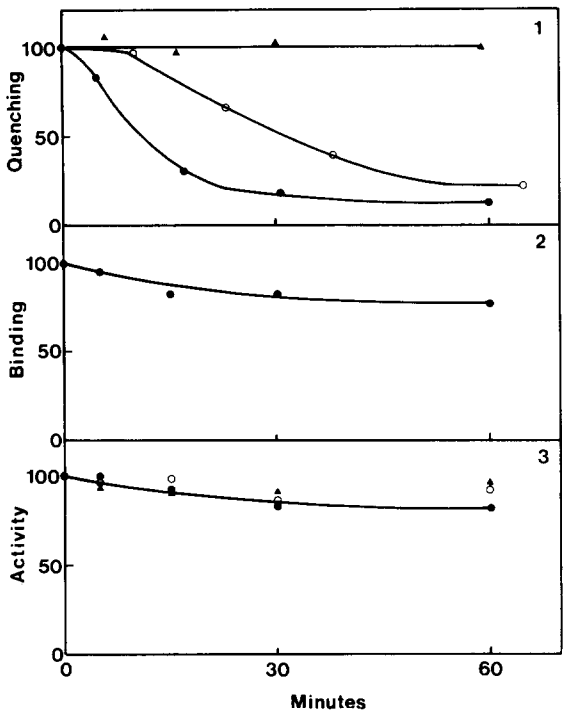

Fig. 7. Time-course of changes in ATPase activity, binding to stripped membrane vesicles, and reconstitution of ATP-energized quenching of the fluorescence of quinacrine, with ECF_1 preparations from centrifuged Sephadex G-50 columns equilibrated with triethanolamine buffer with (triangles) or without EDTA (solid circles). The open circles were obtained for enzyme which on emergence from a column equilibrated without EDTA had been supplemented with 10 mM ATP/5 mM $MgCl_2$. Binding activity and quenching are expressed as a percentage of the zero-time value, i.e., that given by the sample collected immediately on emergence from the centrifuged column.

cence quenching. A substantial decrease had occurred by 15 min. This loss in ability to reconstitute was not accompanied by any marked decrease in the ability of the enzyme to bind to stripped membrane vesicles (about 50% of the added enzyme was bound) (middle panel), or with loss of hydrolytic activity (lower panel) or of the stimulation of ATP hydrolysis by LDAO (data not shown). The presence of ATP and $MgCl_2$ slowed down but did not prevent the loss of ability to reconstitute fluorescence quenching. EDTA in the column buffer fully protected against loss of this activity.

Examination of SDS-polyacrylamide gels of samples removed at intervals after the column treatment showed that there was progressive formation of αδ crosslinks in enzyme prepared in triethanolamine buffer only (Fig. 6, right panel). This could be correlated with the loss of ability to reconstitute fluorescence quenching. The enzyme prepared by column treatment in the presence of EDTA showed no formation of αδ cross-links (data not shown). This correlates with the retention of reconstitution activity by this preparation. ATP and $MgCl_2$ added to the enzyme prepared without EDTA could not prevent the formation of αδ cross-links. The amount of αδ-cross-linked enzyme was about the same at 60 min in samples with and without added ATP and $MgCl_2$ (data not shown).

Effect of DCCD on rebound αδ-cross-linked ECF_1

Although αδ-cross-linked ECF_1 was bound to the same extent as native enzyme to ECF_1-stripped membrane vesicles, it was of interest to see if it rebound to ECF_0. This was assessed by the retention of sensitivity to DCCD in the membrane-rebound enzyme. Soluble ECF_1 is insensitive to

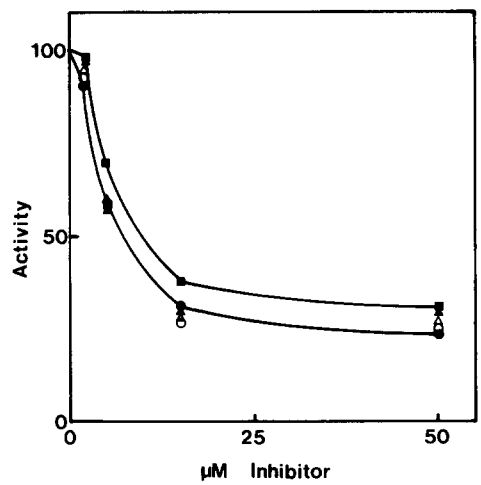

Fig. 8. Inhibition by DCCD of ATPase activity of ECF_1 rebound to stripped membrane vesicles. Open circles, native enzyme; solid circles, solid triangles, open triangles and solid squares were obtained using ECF_1 from a centrifuged Sephadex G-50 column equilibrated with triethanolamine buffer and kept at 20°C for 5, 15, 30 and 60 min, respectively. The reconstituted membrane vesicles were incubated with DCCD at pH 8.5 in 50 mM Hepes-KOH buffer containing 2.5 mM $MgCl_2$ at 37°C for 15 min prior to measurement of ATPase activity. The protein concentration during incubation with DCCD was 0.2 mg/ml. Activity is expressed as a percentage of the activity found in the absence of DCCD.

DCCD at pH 8.5. Inhibition of the ATPase activity of ECF_1 in the membrane-bound enzyme is due to the reaction of DCCD with ECF_0. This requires the correct alignment of ECF_1 on ECF_0 in order that the conformational change responsible for this inhibition can be transmitted from ECF_0 to ECF_1 [29–31].

Samples were taken at intervals from an ECF_1 preparation which had been passed through the Sephadex G-50 column in triethanolamine buffer to generate αδ-cross-linked enzyme. The samples were rebound to stripped membrane vesicles and the extents of inhibition of ATPase activity at various concentrations of DCCD were measured. As shown in Fig. 8, all samples, except the one incubated for 60 min, had similar sensitivities to DCCD as that shown by the native enzyme. The 60 min sample was slightly less sensitive. It is concluded that the αδ-cross-linked ECF_1 rebinds correctly to ECF_0 in stripped membrane vesicles.

Cross-linking

The pattern of crosslinked products formed by 3,3′-dithiobis(succinimidyl propionate) with αδ-cross-linked ECF_1 were as observed with the native enzyme [21]. Both enzymes yielded cross-linked αα, αβ, βγ, βε and γε dimers (results not shown).

Discussion

In this study two experimental conditions lead to the formation of a disulfide cross-link between α and δ subunits of ECF_1. Both depended on the removal of the protective thiol (dithiothreitol) in the absence of EDTA. This was accomplished either by sucrose gradient centrifugation or, more conveniently and rapidly, by gel filtration through Sephadex G-50 by the centrifuged column technique of Penefsky [23]. Presumably, both techniques lead to oxidation of suitably placed sulfhydryl groups on the α and δ subunits with the formation of a disulfide bridge. Oxidation was likely catalyzed by a contaminating divalent cation in the buffer, since it could be prevented by EDTA. Neither technique lead to complete conversion of the enzyme to the αδ-cross-linked form, but 75–80% conversion could be attained readily.

ECF_1 from strain ML308-225 did not readily form αδ cross-links. The reason for this is unclear, although the enzyme was somewhat more stable than that from K12 strains. For example, it much less readily lost subunits on sucrose gradient centrifugation in the absence of ATP.

The αδ-cross-linked enzyme resembled the native enzyme in many of its properties. The enzyme sedimented in a sucrose gradient similarly to the native enzyme. Both enzymes had similar specific activities, and both were stimulated to the same extent by LDAO. Both enzymes bound equally well to ECF_1-stripped membrane vesicles and the ATPase activities of the bound enzymes were inhibited by DCCD to the same extent. Chemical cross-linking of both enzymes with 3,3′-dithiobis(succinimidyl propionate) gave identical cross-linked products. The dissociation constants for ADP and for aurovertin D from the ADP · ECF_1 · aurovertin D and ECF_1 · aurovertin D complexes, respectively, were similar for native and αδ-cross-linked ATPases.

The only difference seen between native and αδ-cross-linked ECF_1 was in their different abilities to reconstitute NADH oxidation- or ATP hydrolysis energized quenching of the fluorescence of quinacrine. Although there is some disagreement about what is being measured by this reaction [32,33], fluorescence quenching can be used as a qualitative measure of proton translocation [33,34]. It is also a measure of the leakiness of vesicle membranes to protons. Thus, the stripping of ECF_1 from vesicles results in the loss of the NADH oxidation-dependent quenching of quinacrine fluorescence, since the membranes can no longer support a proton gradient. In the present experiments the inability of NADH and ATP to quench with vesicles reconstituted with the αδ-cross-linked enzyme was probably due to the inability of the cross-linked enzyme to prevent passive proton movement through ECF_0. If this had been prevented by the binding of ECF_1, then NADH oxidation would have resulted in fluorescence quenching. The retention of DCCD-sensitivity by the membrane-bound αδ-cross-linked ECF_1 suggests that this enzyme is interacting with ECF_0 in the same way as the native enzyme. Therefore, the most plausible explanation of the leakiness to protons of the vesicle system containing rebound αδ-cross-linked ECF_1 is that cross-linking of δ to

α affects the normal proton pathway between ECF_0 and ECF_1, and not that protons leak through an improperly sealed ECF_0 proton channel. A further implication of these results is that the δ subunit is the most likely route for protons between ECF_0 and ECF_1, and that the conformational changes involved in the transmission of DCCD-sensitivity from ECF_0 to ECF_1 do not involve this subunit. In support of the latter idea, is evidence from other sources for interactions between ECF_1 and ECF_0 which do not involve the δ subunit [35,36].

The inhibitory effect of a mutation in the α subunit of ECF_1 on ATPase activity and other properties of the enzyme has been taken as evidence of the importance in the reaction mechanism of the ATPase of cooperative interactions between α subunits and active site-bearing β subunits [6,18]. Therefore, it was interesting to find that many of the catalytic properties of the αδ-cross-linked ECF_1 resembled those of the native enzyme. It would have been expected that cross-linking between the δ subunit and an α subunit would have affected the conformational behaviour of the α subunit. This suggests that the portion of the α subunit cross-linked to the δ subunit is not directly involved in cooperative interactions with the β subunit or that the α subunits of the enzyme behave asymmetrically so that only those α subunits not interacting with the δ subunit are directly involved in the catalytic process. An asymmetric model for the ATPase based on other evidence has been proposed previously [22].

A final point emerging from the studies described in this paper concerns the mechanism of the activation of ATPase activity by LDAO. Lötscher et al. [28] suggested that activation was due to the LDAO-induced movement of the inhibitory ε subunit from an active site on a β subunit. This was based on the loss of EDC-mediated cross-linking of β to ε in the presence of LDAO. However, we show here that the ATP and $MgCl_2$ present in the ATPase assay during the measurement of LDAO activation effectively counteract the inhibitory effect of LDAO on the EDC cross-linking of β to ε. Thus, ε does not necessarily change its structural relationship to β in the course of LDAO activation of the ECF_1. Another explanation for LDAO activation must be sought.

Acknowledgements

This work was supported by a grant from the Medical Research Council of Canada. We thank Dr. R.D. Simoni (Stanford University) for the gift of strain LE392pRPG54.

References

1 Futai, M. and Kanazawa, H. (1983) Microbiol. Revs. 47, 285–312
2 Senior, A.E. and Wise, J.G. (1983) J. Membrane Biol. 73, 105–124
3 Bragg, P.D. (1984) Can. J. Biochem. Cell Biol. 62, 1190–1197
4 Vignais, P.V. and Satre, M. (1984) Mol. Cell. Biochem. 60, 33–70
5 Dunn, S.D. and Heppel, L.A. (1981) Arch. Biochem. Biophys. 210, 421–436
6 Bragg, P.D., Stan-Lotter, H. and Hou, C. (1982) Arch. Biochem. Biophys. 213, 669–679
7 Schäfer, H.-J. and Dose, K. (1984) J. Biol. Chem. 259, 15301–15306
8 Dunn, S.D. and Futai, M. (1980) J. Biol. Chem. 255, 113–118
9 Maeda, M., Kobayashi, H., Futai, M. and Anraku, Y. (1976) Biochem. Biophys. Res. Commun. 70, 228–234
10 Bragg, P.D. and Hou, C. (1977) Arch. Biochem. Biophys. 178, 486–494
11 Wise, J.G. and Senior, A.E. (1985) Biochemistry 24, 6949–6954
12 Issartel, J.-P., Lunardi, J. and Vignais, P.V. (1986) J. Biol. Chem. 261, 895–901
13 Bragg, P.D., Davies, P.L. and Hou, C. (1973) Arch. Biochem. Biophys. 159, 644–670
14 Sternweis, P.C. and Smith, J.B. (1977) Biochemistry 16, 4020–4025
15 Sternweis, P.C. (1978) J. Biol. Chem. 253, 3123–3128
16 Duncan, T.M. and Senior, A.E. (1985) J. Biol. Chem. 260, 4901–4907
17 Noumi, T., Futai, M. and Kanazawa, H. (1984) J. Biol. Chem. 259, 10076–10079
18 Wise, J.G., Latchney, L.R. and Senior, A.E. (1981) J. Biol. Chem. 256, 10383–10389
19 Foster, D.L. and Fillingame, R.H. (1982) J. Biol. Chem. 257, 2009–2015
20 Gunsalus, R.P., Brusilow, W.S.A. and Simoni, R.D. (1982) Proc. Natl. Acad. Sci. USA 79, 320–324
21 Bragg, P.D. and Hou, C. (1986) Arch. Biochem. Biophys. 244, 361–372
22 Bragg, P.D. and Hou, C. (1980) Eur. J. Biochem. 106, 495–503
23 Penefsky, H.S. (1977) J. Biol. Chem. 252, 2891–2899
24 Laemmli, U.K. (1970) Nature (London) 227, 680–685
25 Bragg, P.D. and Hou, C. (1975) Arch. Biochem. Biophys. 167, 311–321
26 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254

27 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
28 Lötscher, H.-R., De Jong C. and Capaldi, R.A. (1984) Biochemistry 23, 4140–4143
29 Sebald, W. and Hoppe, J. (1981) Curr. Top. Bioenerg. 12, 1–64
30 Fillingame, R.H., Peters, L.K., White, L.K., Mosher, M.E. and Paule, C.P. (1984) J. Bacteriol. 158, 1078–1083
31 Penefsky, H.S. (1985) Proc. Natl. Acad. Sci. USA 82, 1589–1593
32 Chow, W.S. and Barber, J. (1980) Biochim. Biophys. Acta 589, 346–352
33 Huang, C.-S., Kopacz, S.J. and Lee, C.-P. (1983) Biochim. Biophys. Acta 722, 107–115
34 Casadio, R. and Melandri, B.A. (1985) Arch. Biochem. Biophys. 238, 219–228
35 Aris, J.P. and Simoni, R.D. J. Biol. Chem. 258, 14599–14609
36 Loo, T.W., Stan-Lotter, H., McKenzie, D., Molday, R.S. and Bragg, P.D. (1983) Biochim. Biophys. Acta 733, 274–282