Biochimica et Biophysica Acta 890 (1987) 97–105
Elsevier

BBA 42066

Escherichia coli F_1 ATPase is reversibly inhibited by intra- and intersubunit crosslinking: an approach to assess rotational catalysis

Rajendra P. Kandpal and Paul D. Boyer

Molecular Biology Institute and Department of Chemistry and Biochemistry, University of California, Los Angeles, CA 90024 (U.S.A.)

(Received 2 September 1986)

Key words: F_1-ATPase; ATPase crosslinking; Rotational catalysis; ATPase conformation; (*E. coli*)

Reaction of the multisubunit F_1 ATPase from *Escherichia coli* (EF_1) with a bifunctional cleavable crosslinker, 3,3′-dithiobis(succinimidylpropionate) (DSP), has been used to explore the possibility that during catalysis a rotational movement of catalytic subunits relative to noncatalytic subunits occurs. The premise is that such rotational catalysis is tenable if intersubunit crosslinking of a major subunit with one of the minor subunits inhibits the enzyme activity and if upon cleavage of the crosslinks, the enzyme regains activity. The results presented in this paper show that crosslinking of about 5–6 reactive groups on EF_1 with DSP is accompanied by a loss of 2/3 of the enzyme activity. Both intra- and intersubunit crosslinks are formed. The most prominent intersubunit crosslinks are those of γ and δ subunits with the α subunit. Nearly complete recovery of activity can be attained by cleaving the disulfide bond in the crosslinker with dithiothreitol. Because the chemical modification of enzyme groups remains after the crosslinker is cleaved, the loss in activity before cleavage can be ascribed to conformational restraints. The results show that catalysis by the EF_1 ATPase is highly sensitive to the restrictions of crosslinking, and are consistent with the view that catalysis is accompanied by appreciable movements of the major subunits with respect to the minor subunits, as suggested for rotational catalysis.

Introduction

The F_1 ATPases from bacteria, chloroplasts and mitochondria consist of five subunits with a stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon$, with the catalytic site likely residing on the β subunit (see for reviews, Refs. 1 and 2). The binding change mechanism for ATP synthesis [3] has been proposed to involve sequential participation of the catalytic sites. All three β subunits are regarded as being functionally equivalent but as being in three different conformations at any one time. Recent papers from several laboratories [4–6] add to the evidence for structural asymmetry of the β subunits of the enzyme under conditions when it is not turning over.

Prominent interactions occur between the γ subunit and the α and β subunits as shown by the requirement of all three subunits for catalysis (see review, Ref. 7), and the demonstrations that modifications of –SH groups on the γ subunit of CF_1 ATPase markedly affect catalysis [8]. Measurements in this laboratory of the species of $[^{18}O]P_i$

Abbreviations: F_1, coupling factor one ATPase; EF_1 ATPase, F_1 ATPase from *Escherichia coli*; Mops, 4-morpholinepropanesulfonic acid; DSP, 3,3′-dithiobis(succinimidylpropionate); SDS, sodium dodecyl sulfate; DMSO, dimethylsulfoxide; P_i, inorganic phosphate; DTT, dithiothreitol.

Correspondence: Dr. Paul D. Boyer, Department of Chemistry and Biochemistry, University of California, Los Angeles, CA 90024, U.S.A.

formed from ^{18}O-labeled ATP during ATP hydrolysis [9], or of [^{18}O]ATP formed from [^{18}O]P_i during ATP synthesis [10] demonstrate that every participating catalytic site is performing catalysis in a closely similar or identical manner. The sequence data for the F_1 ATPases [11] show lack of any tripartite symmetry of the γ subunit. Thus, certain groups or regions on the γ subunit must interact with the β subunit, either directly or through the α subunit, to influence the catalytic behavior of the β subunits. For the three β subunits to pass through equivalent states during catalysis, the β subunits would need to sequentially change their positions in the enzyme complex in relation to the γ subunit.

Such considerations led to the suggestion in 1981 [12,13] of a "rotational catalysis" for the ATP synthase and F_1 ATPases involving a rotational change in the position of the β subunits relative to a core of the minor subunits. Subsequently, based on structural considerations, Cox et al. advanced a similar proposal [14], Oosawa and Hayashi have proposed a mechanism for a rotational model [15], and Mitchell has suggested a model in which α and β subunits spin in place [16]. The Boyer and the Cox proposals explain how the β subunits can maintain interactions with minor subunits, yet pass through identical conformations during catalysis.

Experimental approaches that have the potential for proving whether or not rotational catalysis occurs are difficult to formulate. The approach used for the present paper had the possibility of proving that rotational catalysis very likely did not occur or of adding to the findings consistent with such catalysis. The latter result was obtained.

If rotational catalysis occurs, a chemical crosslink between the β and γ subunits would be expected to stop catalysis. If catalysis continued with such a crosslink, rotational catalysis would be untenable. If the lost activity is regained when the crosslink is cleaved, then spatial restrictions and not chemical modification of groups could be regarded as the reason for loss of catalytic capacity. Such behavior would provide evidence consistent with rotational catalysis. Although a considerable number of crosslinking experiments have been performed with F_1 ATPases [17–23], and loss of catalytic activity has resulted [19,20,23], the use of cleavable crosslinkers in an approach that would test rotational catalysis has not to our knowledge been undertaken.

The studies reported here made use of a cleavable crosslinker, DSP. The inhibition of over 50% of the catalytic activity of EF_1 ATPase was found to be nearly completely reversible by cleavage of the crosslinker. The activity loss thus resulted from intersubunit and intrasubunit crosslinking and not from extensive derivatization of reactive groups, likely $-NH_2$ groups.

Experimental procedures

Materials

ATP, NADH, pyruvate kinase, lactate dehydrogenase, ε-amino caproic acid, *p*-aminobenzamidine, and dithiothreitol were supplied by the Sigma Chemical Co. Sepharose CL-6B and DEAE-Sepharose CL-6B were obtained from Pharmacia Fine Chemicals. DSP was a product of Pierce Chemical Co. *E. coli* strain AN 1460 was obtained from Dr. A.E. Senior of the University of Rochester.

Methods

Purification of EF_1 ATPase. E. coli strain AN 1460 was grown to mid-exponential phase and the cells were stored at −80°C.

The membrane preparation and enzyme purification were essentially carried out by the method of Senior et al. [24], except for minor changes in the F_1 purification procedure. The eluate from DEAE-Sepharose CL-6B was precipitated with 65% saturated ammonium sulfate. The precipitate was dissolved in 50 mM Tris buffer (pH 7.4), containing 10% (v/v) glycerol/2 mM EDTA/1 mM ATP/1 mM DTT/40 mM ε-amino caproic acid. The solution was then applied to a column of Sepharose CL-6B (2.5 cm × 90 cm), the eluted fractions containing highest specific activity were pooled, and the enzyme was stored as an ammonium sulfate suspension at 4°C.

ATPase assay. The enzyme activity was monitored by a coupling assay [25] after incubation in dilute solution. The specific activity of EF_1 increases as concentration is lowered as a result of dissociation of the ε subunit from the complex [26,27]. The enzyme was assayed at a concentra-

tion of around 0.1 μg/ml after preincubation at 30°C for 20 min in a mixture consisting of 30 mM Tris-acetate (pH 7.6)/30 mM potassium acetate/3 mM ATP/40 μg per ml pyruvate kinase/20 μg per ml lactate dehydrogenase/0.2 mM NADH. The reaction was initiated by adding an aliquot of Mg-acetate (final concentration, 5 mM) and activity was measured by determining the decrease in the absorbance of NADH at 340 nm. The specific activity of the enzyme was expressed as μmol product formed/min per mg protein. Protein was determined by the method of Lowry et al. [28], using bovine serum albumin as standard.

Crosslinking with DSP. Two procedures were used for crosslinking the enzyme. In one, an aliquot of the ammonium sulfate suspension was pelleted, dissolved in 100 mM Mops-Tris buffer (pH 7.0) and passed through a centrifuge-Sephadex column [29] equilibrated with the same buffer. The desalted enzyme was crosslinked in a mixture containing 100 mM Mops-Tris (pH 7.0)/20 mM ATP. An aliquot of DSP (20–40 mM stock in dimethyl sulfoxide) was added such that the final DMSO concentration was less than 10% (v/v). The reaction was allowed to proceed for 5 min at room temperature (22–25°C) or as indicated in various experiments and then passed through a centrifuge-Sephadex column. The eluate was used for the assay of ATPase activity and electrophoresis.

Alternatively, an aliquot of the ammonium sulfate suspension was pelleted, dissolved in 50 mM phosphate buffer (pH 7.0) and passed through a centrifuge-Sephadex column equilibrated with the same buffer. The crosslinking reaction was carried out in a reaction mixture containing 50 mM phosphate buffer (pH 7.0) and 20 mM ATP at 10°C, as described in the preceding paragraph.

The control enzyme was similarly treated with only DMSO and processed as described for experimental samples.

Cleavage of crosslinks with DTT. The crosslinked enzyme was incubated with 33 mM DTT and 33 mM ATP at 37°C for 30 min and then assayed for enzyme activity. Because of the high dilution for assay, the concentration of ATP and other constituents during assay were essentially those of the assay mix. The control (uncrosslinked) enzyme was treated in the same manner.

Gel electrophoresis. One-dimensional SDS-polyacrylamide gel electrophoresis was carried out with either a 12% or 15% uniform or 3–22% gradient gel by the method of Laemmli [30]. A stacking gel (5%) was used with the uniform polyacrylamide gels. The stacking gel was omitted for the gradient gel and a comb was inserted immediately after pouring gradients, and the gel was allowed to polymerize. The crosslinked samples were prepared in a sodium dodecyl sulfate sample buffer lacking 2-mercaptoethanol. 30–50 μg protein was applied to the wells and electrophoresis was carried out at 50 mA per slab for 3 h. The gel was stained with Coomassie Blue R overnight.

For the second dimension, the gel strip (1 cm wide) was cut out from the one-dimensional gel and placed horizontally over a 5% stacking gel of a 0.75 mm thick 15% acrylamide gel. The strip was surrounded by 1% agarose in 125 mM Tris-HCl buffer (pH 8.5) containing 0.1% (w/v) sodium dodecyl sulfate and 2% (v/v) 2-mercaptoethanol, and run at a current of 50 mA per slab.

Quantitation of subunits in the control and crosslinked enzyme. The relative amounts of different subunits were estimated by excising the bands corresponding to α, β, γ, δ and ε subunits, extracting the dye in 1 ml DMSO (100%) for over 12 h at room temperature and determining the absorbance in DMSO of the extract at 595 nm. The same procedure was used for quantitating the subunits in the second dimension gel, obtained after DTT cleavage.

To test the dye extraction method, various amounts of EF_1 were put through the separation and extraction procedure. The absorbance was found to increase linearly for individual subunits over a range of 6–50 μg of applied EF_1. All the extractions were carried out with duplicate samples.

Results

Time-course and buffer dependency of the reaction with DSP

The time-course of the reaction in 100 mM Mops-Tris buffer or 50 mM phosphate buffer at pH 7.0 is shown in Fig. 1. The results show that the reaction reached completion in about 5 min when carried out in Mops-Tris buffer at room

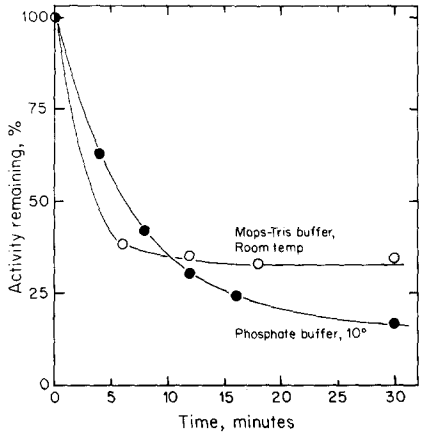

Fig. 1. Time-course for inactivation of EF_1 ATPase with DSP. O——O, inactivation in Mops-Tris buffer. To EF_1 ATPase (1.54 mg/ml) in 100 μl of a reaction mixture at room temperature containing 100 mM Mops-Tris buffer (pH 7.0) and 20 mM ATP was added 2 μl of DSP in DMSO (DSP concentration, 0.5 mM final). The reaction mixture was vortexed immediately and incubated at room temperature (22–25°C). ●——●, inactivation in phosphate buffer. EF_1 ATPase (1.01 mg/ml) in 100 μl of a reaction mixture at 10°C containing 50 mM phosphate buffer (pH 7.0) and 20 mM ATP was treated with DSP as indicated for the reaction with Mops-Tris buffer, except incubation was at 10°C. Aliquots were drawn at times indicated in the figure and assayed as described in Experimental procedures.

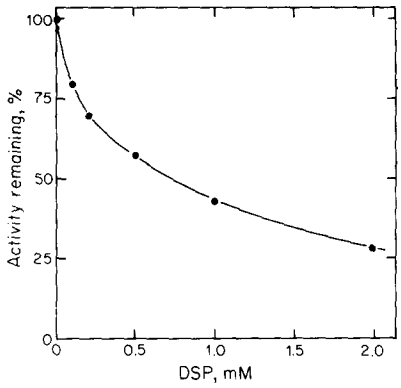

Fig. 2. Effect of DSP concentration on EF_1 ATPase activity. To EF_1 ATPase (0.62 mg/ml) in 100 μl of a reaction mixture containing 50 mM phosphate buffer and 20 mM ATP at pH 7.0, was added 5 μl of DSP in DMSO (final concentration of DSP as indicated). The mixture was vortexed immediately and incubated at 10°C for 5 min. The reaction mixture was then passed through a centrifuge-Sephadex column and the eluate was used to assay enzyme activity as described in Experimental procedures.

temperature. A slower reaction occurred in phosphate buffer at 10°C, however, the extent of inactivation was greater than in Mops-Tris buffer, suggesting that the DSP reacts with Tris, thereby reducing its effective concentration in the reaction mixture. More variability was encountered when Tris buffer was used. This could also be a reflection of a reaction of the reagent with the Tris buffer, if, for example, there are rapid competing reactions as the reagent added in DMSO is being diluted.

Effect of DSP concentration on activity loss

Fig. 2 shows the effect of a 5 min exposure to varying concentration of DSP on the activity of the enzyme. The crosslinking in these experiments was carried out in PO_4 buffer at 10°C. The activity loss approximated a first-order reaction course.

Reversible loss of enzyme activity by reaction with DSP

When the EF_1 ATPase was treated with DSP, subunit crosslinking similar to that reported by Bragg and Hou [20] was observed, but with less extensive crosslinking as anticipated by our use of lower DSP concentrations.

Table I shows representative experiments showing loss of activity due to enzyme modification by DSP in 100 mM Mops-Tris buffer. The results

TABLE I

EFFECT OF CROSSLINKING AND CLEAVAGE OF CROSSLINKS ON THE ACTIVITY OF EF_1 ATPase

EF_1 ATPase (1.1–4.6 mg/ml) was modified with the indicated concentrations of DSP, in a reaction mixture containing 100 mM Mops-Tris and 20 mM ATP at pH 7.0 for 5 min at room temperature. The reaction was stopped by passing the mixture through a centrifuge-Sephadex column. The eluate was used for ATPase assay. The crosslinks were cleaved with dithiothreitol (DTT) in the presence of ATP at 37°C as described in the Experimental procedures section. The results are means ± standard deviation of four experiments. Each assay was carried out in duplicate.

Concentration of DSP (mM)	Activity (%)	Activity after DTT treatment (%)
0	100	100 ± 5
2	29 ± 12	85 ± 4
3.33	29 ± 3	70 ± 12

indicate that as much as 70% activity was lost at a DSP concentration of 3.33 mM. A major portion of the lost activity could be recovered on cleaving the crosslinks with dithiothreitol in the presence of ATP. Other experiments showed that the presence of ATP in the modification mixture was essential for reversible inactivation. When modification was carried out in the absence of ATP, very little activity was regained after dithiothreitol cleavage of the crosslinks (data not shown). Also, the presence of ATP was required for optimal regain of activity upon cleavage of crosslinks with dithiothreitol.

Correlation of subunit crosslinking with enzyme inactivation

To find whether activity loss might be attributed to any particular intersubunit crosslinks, the subunits crosslinked were identified by gel electrophoresis. Intersubunit crosslinking was evident after DSP reaction as shown by the appearance of higher molecular-weight species (Fig. 3). The higher molecular-weight crosslinks were not attributable to intermolecular crosslinking because their appearance was independent of enzyme concentration. Also, the electrophoresis of the crosslinked enzyme on a nondissociating gel (no SDS) revealed the absence of intermolecular crosslinking. In this gel, the undissociated enzyme migrated as a single major band.

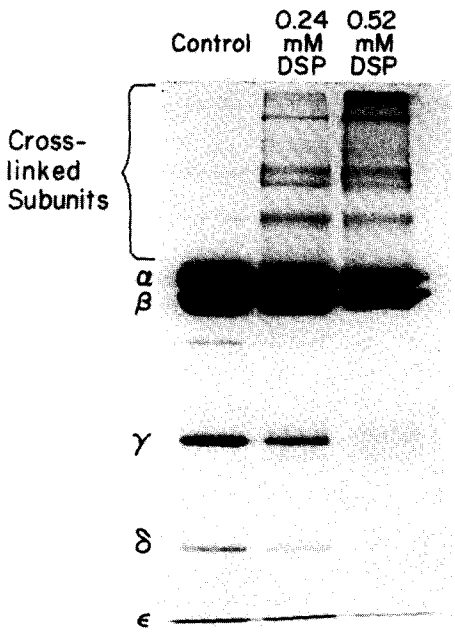

Fig. 3. Gel electrophoresis patterns after crosslinking. EF_1 ATPase (1.9 mg/ml) in a 100 µl volume was incubated with 0.24 or 0.52 mM DSP in 100 mM Mops-Tris buffer, containing 20 mM ATP at pH 7.0, and at room temperature for 5 min as described in Experimental procedures. The enzyme retained 69% and 29% activity, respectively, and regained 100% and 87% activity after dithiothreitol-cleavage. 42 µg (control), 38 µg (modified with 0.24 mM DSP) and 31 µg (modified with 0.52 mM DSP) of protein were applied as indicated to a 12% SDS-polyacrylamide gel, with 5% stacking gel, and electrophoresed at 50 mA for 3 h before staining.

Experimental results of crosslinking in Tris buffer are presented in Table II. The amount of noncrosslinked subunit remaining after reaction with DSP is expressed as the % of the total original amount of subunit present. The data show that the extent of crosslinking of γ and δ subunit was about equal to the loss of activity, while the extent of crosslinking of the α and β subunits was much less than the activity loss. The γ and δ subunits that were crosslinked were present in bands corresponding to greater apparent M_r than underivatized α and β subunits showing that they were crosslinked to the larger subunits.

In one of these experiments the amounts of all the five subunits remaining uncrosslinked were compared with native enzyme. The results are presented in part B of Table II. They show that crosslinking of 33% of the α subunit or 11% of the β subunit is accompanied by 71% loss of activity. The preferential crosslinking of the α subunit suggests that the principal crosslinks of the γ and δ subunits were to the α subunit. γ–δ subunit crosslinks were not observed. Actiivty loss was somewhat greater than the crosslinking of the γ or δ subunits to the larger subunits. There was little crosslinking of the ε subunit with any other subunit.

Experimental results of subunit crosslinking in phosphate buffer are presented in Table III. The loss of activity and extent of crosslinking were more pronounced relative to the reaction in Mops-Tris buffer. Crosslinking was 61%, 28%, 65%, 69%, 32% for α, β, γ, δ and ε subunits, respectively, when modification was carried out for 15 min (Table IIIA). Electrophoresis of the crosslinked species in a second dimension after di-

TABLE II

EFFECT OF DSP TREATMENT ON THE EXTENT OF INTERSUBUNIT CROSSLINKING AND EF_1 ATPase ACTIVITY

EF_1 ATPase (1.9–2.9 mg/ml) was modified with the indicated concentration of DSP. The reaction mixture containing 100 mM Mops-Tris/20 mM ATP at pH 7.0 and EF_1 ATPase was added to an aliquot (5 μl) of DSP in dimethylsulfoxide and vortexed immediately. Incubation was carried out for 5 min at room temperature. The control enzyme designated as unmodified was similarly treated with an aliquot of dimethylsulfoxide. The reaction was stopped by passing the mixture through a centrifuge-Sephadex column. The eluate was used for enzyme assay and electrophoresis. Electrophoresis and quantitation of individual subunits were carried out as described in the Experimental procedures section. The results are the mean of three experiments. All the determinations and assays for each experiment were carried out in duplicate. The results in part B of the table represent a typical experiment, where amounts of all the five subunits were determined. DTT, dithiothreitol.

IIA

Concentration of DSP (mM)	Activity (%)	Activity after DTT treatment (%)	Subunits not crosslinked (%)		
			α + β	γ	δ
0	100	118 ± 3.7	100	100	100
0.24	73.5 ± 7.2	114 ± 2.0	84 ± 3	60 ± 5	69 ± 8
0.52	33.6 ± 4.5	91 ± 12.0	71 ± 7	34 ± 8	41 ± 3

IIB

Enzyme	Activity (%)	Activity after DTT treatment (%)	Subunits not crosslinked (%)				
			α	β	γ	δ	ε
Unmodified	100	120	100	100	100	100	100
Modified	29	76	67	89	44	44	96

thiothreitol-cleavage showed that αβ, αα, αγ, αδ and β_γ crosslinks were formed.

Occurrence of intersubunit crosslinks

The first dimension electrophoresis and determination of uncrosslinked subunits by dye extraction revealed that a larger fraction of the α subunit than of the β subunit was crosslinked. To ascertain which subunits were crosslinked, the first dimension gel strip was run in a second dimension after DTT-cleavage. The bands corresponding to α, β, γ, δ and ε subunits in the uncrosslinked, as well as crosslinked species, were determined. The results are expressed as the percent of unmodified (native) enzyme subunits (Table IIIB). In the second dimension, the uncrosslinked subunits move along a diagonal path, while the subunits arising from cleavage of the crosslinked species move away from this diagonal. About equal amounts of native and modified enzyme preparations were applied to the gel whenever a comparison of the relative abundance of subunits was made. The results presented in part B of Table III confirm that the individual subunits indicated as lost in part A appear in the crosslinked species. The extent of crosslinking reported in part B agrees reasonably well with that presented in part A. A greater proportion of α subunits than of the β subunits was observed in the crosslinked species.

Although as much as 20–30% reduction in the amount of uncrosslinked ε subunit was observed in some experiments, no ε subunit was found in the cleavage products of the crosslinked species of mass greater than 55–60 kDa. Some intramolecular crosslinks in the ε subunit may have caused a change in its mobility, without crosslinking it to a major subunit, or it was dispersed in crosslinked products so that its abundance was too low to show up in the second dimension.

This left open the question whether the inhibition of activity upon crosslinking might result from binding of the ε subunit to the enzyme in a manner that retained the inhibitory effect of the subunit upon dilution of the enzyme to attain

TABLE III

EFFECT OF DSP TREATMENT ON THE EXTENT OF INTERSUBUNIT CROSSLINKING AND EF_1 ATPase ACTIVITY

To EF_1 ATPase (1.0–1.45 mg/ml) in a 400 μl volume containing 50 mM phosphate buffer and 20 mM ATP at pH 7.0, was added a 10 μl aliquot of DSP in dimethylsulfoxide (final concentration, 0.5 mM) and the reaction mixture vortexed immediately. Incubations were at 10°C. Aliquots were withdrawn at times indicated and passed through centrifuge-Sephadex columns. The eluate was used for enzyme assays and electrophoresis. The results are means of three experiments. The determinations for each experiment were carried out in duplicate. Part B of the table presents the percentages of the subunits in uncrosslinked and crosslinked fractions of the modified preparation. The enzyme modified with DSP for 15 min was run in SDS-polyacrylamide gel electrophoresis. This lane was then run in the second dimension after dithiothreitol (DTT) cleavage as described in the Experimental procedures section. The amounts of subunits are expressed as percent of unmodified preparation presented in the first row of part A. The results are mean of three experiments.

A. One-dimensional electrophoresis

Sample	Incubation time with DSP	Activity	Activity after DTT-treatment	Fraction of subunit not crosslinked				
				α	β	γ	δ	ε
	(min)	(%)	(%)	(%)	(%)	(%)	(%)	(%)
1	0	100	106 ±6	100	100	100	100	100
2	5	67.5±4.9	94.5±2.5	69±3	89± 6	68±2	72±10	77±11
3	10	53.0±1.0	85.5±6.5	63±7	88± 5	54±1	70± 3	76± 5
4	15	41.6±2.5	83.3±4.5	39±8	72±11	35±9	31± 4	68± 8

B. Second dimension of electrophoresis for sample 4

Subunit measurement	Subunit				
	α	β	γ	δ	ε
Recovered from uncrosslinked portion of gel [a]	32±4	58±5	25±7	31±14	51±7
Recovered from crosslinked portion of gel [a]	44±6	17±4	42±7	33± 8	–
Total recovered from gel [a]	76	75	67	64	–
Fraction not crosslinked	0.42	0.77	0.37	0.48	–

[a] As % of subunits found in sample 1, part A, with no DSP treatment.

maximum specific activity. When the noncrosslinked enzyme was assayed at a concentration of 1.5 μg/ml, no effect of subunit dissociation was apparent and the specific activity was about 1/3 of that observed when assayed at 0.1 μg/ml (where specific activity is maximal [26,27]). When the crosslinked enzyme was assayed at a concentration of 1.5 μg/ml, the inhibition produced was similar to that noted when assayed at 0.1 μg/ml. Hence, the inhibition upon crosslinking can be attributed to derivatization of other subunits and not to lack of ε subunit dissociation.

Extent of derivatization and possible intramolecular crosslinks

To assess the extent of derivatization and of possible intramolecular crosslinks in the major α and β subunits, samples were analyzed by 2-D gel electrophoresis. After cleavage of the –S–S bond by thiol, samples were exposed to isoelectric focusing in one dimension, then separated by SDS-polyacrylamide gel electrophoresis in a second dimension. Reaction with the DSP would be expected to remove a positive charge of an amino group, and thus the subunits that have reacted with one or more succinimidyl groups of the reagent will be revealed as one or more spots migrating separately from the unmodified subunit. After reaction with DSP under the conditions required to inhibit about 2/3 of the catalytic activity, the α subunit no longer migrated primarily as a single spot, but now displayed about equal amounts of five spots as expected for reaction with up to 5 or 6 NH_2 groups for the most derivatized spot. The

β subunit showed migration patterns consistent with derivatization up to 3 or 4 NH_2 groups. It was estimated that an average of 4–6 groups, likely NH_2, on the α and β subunits of each enzyme had reacted. Reaction of less than one group per α or β subunit would suffice to account for the extent of intersubunit crosslinking reported in Table III. The balance of the reacted groups could be singly derivatized with the reagent, the other end of the reagent having reacted with water. The absence of any appreciable intrasubunit crosslinking is indicated by the migration in SDS-polyacrylamide gel electrophoresis of the α and β subunits that had not formed intersubunit crosslinks. Such subunits migrated identically with those of unreacted enzyme (Fig. 1). Intrasubunit crosslinks could readily retard protein unfolding and lessen SDS binding, resulting in changes in migration patterns. But the data do not rule out intrasubunit crosslinks. Each intrasubunit crosslink requires the reaction of two groups on the subunit, and the α subunits could have an average of about two and the β subunits an average of about one such crosslink.

Discussion

The results presented in this paper show the following points. (1) Treatment of EF_1 with DSP results in electrophoretically distinguishable intersubunit crosslinks accompanied by loss of catalytic activity. (2) The loss of activity of the modified enzyme results principally from conformational restraints of crosslinking and not from chemical modification of reactive groups. (3) The extent of crosslinking of α or β subunits is less than the loss of enzyme activity, but the extent of crosslinking of γ or δ subunits is about equivalent to or greater than the loss of enzyme activity.

The crosslinking by DSP likely involves reaction with lysine amino groups on nearby peptide chains. Only a few crosslinks need to be inserted to account for the intersubunit crosslinking observed when about 2/3 of the catalytic activity is lost. Such loss of activity is observed when about 0.7 of the γ and δ subunits are crosslinked to major subunits. A reaction of only 2.8 NH_2 groups per mol could give 1.4 crosslinks per mol. An additional 5–6 NH_2 groups appear to be derivatized in the noncrosslinked α and β subunits, and other NH_2 groups on the γ, δ and ε subunits could have reacted without crosslinking. The recovery of activity after cleavage of the cross-links shows that over 5–6 groups, likely NH_2, per mol can be derivatized without appreciable loss of catalytic activity. This, although of interest, is not an unusual finding. What appears unusual is that the tying together of only 2–4 of these nonessential groups in intersubunit crosslinks could be the cause of a pronounced loss in catalytic activity. The activity loss could result from additive effects of several crosslinks, or, alternatively, specific single intersubunit crosslinks might stop catalysis.

It must be emphasized, however, that the loss of catalytic capacity following reaction with DSP could also result from intrasubunit crosslinks. The isoelectric focusing dimension in 2-dimensional gel analysis showed that although intrasubunit crosslinks may have been lacking, it does not rule out that some intramolecular crosslinks might have been present in the inhibited enzyme, mostly in the α subunits. If α or β subunits undergo appreciable conformational changes during catalysis, then either intrasubunit or intersubunit crosslinks might stop catalytic events. In contrast, for rotational catalysis, it seems highly likely that intersubunit crosslinks between portions of subunits with relative motions in opposite directions would block catalysis.

If the intersubunit crosslinks are responsible for the loss in catalytic activity, then the activity loss is most readily attributable to the linking of the γ and δ subunits to major subunits, particularly the α subunit. Such crosslinks could readily inhibit catalysis by preventing essential conformational changes. If, in rotational catalysis, the β subunits moved around a core that included the α subunits, then crosslinking of 1/3 of the β subunits to α, γ or δ subunits would be expected to stop activity. In the experiment reported in Table IIB, insufficient β subunit was crosslinked to account for activity loss on this basis. If the α and β subunits moved together, the extent of crosslinking of the α subunit to minor subunits in all experiments was sufficient to account for the loss of activity. Even if β subunits moved independently of α subunits, both α and β subunits could have considerable conformational change during

catalysis, and crosslinking of α subunits to single-copy subunits could stop ctalysis.

As mentioned in the Introduction, a finding that a crosslink between the γ subunit and the β subunit specifically inhibited catalysis would support the occurrence of a rotational catalysis as proposed by Boyer and by Cox. The present results are consistent with, but do not provide, a critical test of this possibility. We are currently attempting to devise a means of attaining specific β–γ subunit crosslinks as a further test of the feasibility of rotational catalysis.

The suggestion that the binding change mechanism proceeds with sequential participation of catalytic sites such that the β subunits have the same time-averaged conformations is by no means proven at this stage. The concept of rotational catalysis must be regarded as even more tenuous. However, the results presented here do give strong evidence that major conformational changes accompany catalysis by the F_1 ATPases, and add to the accumulating findings that are consistent with and perhaps best explained by rotational catalysis.

Acknowledgements

We wish to thank John Wise for many valuable suggestions and Z. Xue for performing isoelectric focusing. This study was supported in part by grant GM 11094 from the U.S. Public Health Service and Grant DMB81-00817 of the National Science Foundation.

References

1 Cross, R.L. (1981) Annu. Rev. Biochem. 50, 681–714
2 Senior, A.E. and Wise, J.G. (1983) J. Membrane Biol. 73, 105–124
3 Boyer, P.D. (1979) in Membrane Bioenergetics (Lee, C.P., Schatz, G. and Ernster, L., eds.), pp. 461–479, Addison-Wesley, Reading, MA
4 Lotscher, H.R. and Capaldi, R.A. (1984) Biochem. Biophys. Res. Comm. 121, 331–340
5 Stan-Lotter, H. and Bragg, P.D. (1986) Eur. J. Biochem. 154, 321–327
6 Melese, T. and Boyer, P.D. (1985) J. Biol. Chem. 260, 15398–15401
7 Futai, M. and Kanazawa, H. (1983) Microbiol. Rev. 47, 285–312
8 Nalin, C.M., Beliveau, R. and McCarty, R.E. (1983) J. Biol. Chem. 258, 3376–3381
9 Hutton, R.L. and Boyer, P.D. (1979) J. Biol. Chem. 254, 9990–9993
10 Hackney, D.D. and Boyer, P.D. (1978) J. Biol. Chem. 253, 3164–3170
11 Walker, J.E., Saraste, M. and Gay, N.J. (1984) Biochim. Biophys. Acta 768, 164–200
12 Boyer, P.D. and Kohlbrenner, W.E. (1981) in Energy Coupling in Photosynthesis (Selman, B.R. and Selman-Reimer, S., eds.), pp. 231–240, Elsevier/North-Holland, Amsterdam
13 Gresser, M., Myers, J.A. and Boyer, P.D. (1982) J. Biol. Chem. 257, 12030–12038
14 Cox, G.B., Jans, D.A., Fimel, A.L., Gibson, F. and Hatch, L. (1984) Biochim. Biophys. Acta 768, 201–208
15 Oosawa, F. and Hayashi, S. (1984) J. Phys. Soc. Japan 53, 1575–1579
16 Mitchell, P. (1985) FEBS Lett. 182, 1–7
17 Klein, G., Lunardi, J., Satre, M., Lauquin, G.J.M. and Vignais, P.V. (1977) in Structure and Function of Energy Transducing Membranes (Van Dam, K. and Van Gelder, B.F., eds.), pp. 283–294, Elsevier/North-Holland Biomedical Press, Amsterdam
18 Baird, B.A. and Hammes, G.G. (1976) J. Biol. Chem. 251, 6953–6962
19 Baird, B.A. and Hammes, G.G. (1977) J. Biol. Chem. 252, 4743–4748
20 Bragg, P.D. and Hou, C. (1975) Arch. Biochem. Biophys. 167, 311–321
21 Bragg, P.D. and Hou, C. (1980) Eur. J. Biochem. 106, 495–503
22 Bragg, P.D. and Hou, C. (1986) Arch. Biochem. Biophys. 244, 361–372
23 Chernyak, B.V., Chernyak, Y.Ya., Gladysheva, T.B., Kozhanova, Z.E. and Kozlov, I.A. (1981) Biochim. Biophys. Acta 635, 552–570
24 Senior, A.E., Downie, J.A., Cox, G.B., Gibson, F., Langman, L. and Fayle, D.R.H. (1979) Biochem. J. 180, 103–109
25 Pullman, M.E., Penefsky, H.S., Datta, A. and Racker, E. (1960) J. Biol. Chem. 235, 3322–3329
26 Laget, P.P. and Smith, J.B. (1979) Arch. Biochem. Biophys. 197, 83–89
27 Sternweis, P.C. and Smith, J.B. (1980) Biochemistry 19, 526–531
28 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
29 Penefsky, H.S. (1977) J. Biol. Chem. 252, 2891–2899