CROSSLINKING STUDIES IN OLIGOMYCIN-SENSITIVE ATPase FROM BEEF HEART MITOCHONDRIA

Interrelations of subunits with an M_r 31 000 protein

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1. Introduction

A protein of M_r 31 000 in oligomycin-sensitive (OS)-ATPase is important for oligomycin sensitivity of the preparation [1,2]. Staining intensity of this protein band was decreased by the uncoupler FCCP, control values being restored by subsequent addition of a thiol reagent [3]. Interrelations should exist with the oligomycin-sensitivity conferring protein (OSCP) [4] of ~20 kDa in our preparation.

We have used crosslinking techniques to learn which interdependencies between the 31- and 20-kDa as well as between other subunits can be found. Using tartryl di(glycylazide) (TDGA) [5] we observed crosslinks between proteins of 20 and 34 kDa, between 25–28 and 31 kDa, and between 31 and 34 kDa. Crosslinking at similar sites was obtained with the SH-oxidizing reagent Cu-phenanthroline. It is thus established that the 31- and 20-kDa proteins are connected via the 34-kDa γ-subunit of the OS-ATPase.

We also have found evidence that the 10-, 15- and 31-kDa proteins may exist as dimers. Interactions are also revealed between the 8-, 9- and 13-kDa proteins. Crosslinking with diepoxybutane (DEB) [6] resembled the results obtained with TDGA, and, moreover, showed a decrease in band intensity of the 31-kDa protein band, stabilization of ATPase activity and a concomitant decrease of ATP–P_i exchange reaction.

2. Materials and methods

2.1. *Isolation of beef heart mitochondria*

Beef heart mitochondria were isolated and submitochondrial particles were prepared by the method in [7,8]. Protein estimations were carried out according to [9].

2.2. *Preparation of oligomycin-sensitive ATPase*

OS-ATPase was prepared by the method in [10]. The sucrose gradient centrifugation step in this procedure was omitted. The final pellets of ATPase were resuspended in a buffer containing triethanolamine–HCl 10 mM, EDTA 0.4 mM, $MgSO_4$ 1.0 mM, sucrose 50 mM adjusted with KOH to pH 7.5 and stored at –80°C. Protein was ~30–40 mg/ml.

2.3. *Crosslinking reaction with TDGA [5]*

Immediately after activation of the reagent OS-ATPase (~10 mg/ml) was crosslinked at 40 mM triethanolamine–HCl and pH 8. TDGA was added to a final concentration of 5–40 mM. Incubation at 20°C was carried out for 30 min. Subsequently, the reaction was terminated with excess methylamine. The samples (250 μl) were then prepared for electrophoresis by adding 7.5 mg sodium dodecylsulfate (SDS), 31 μl glycerol, 31 μl sodium phosphate buffer (pH 7) and 4.7 μl mercaptoethanol. Appropriate amounts of sample were layered onto the top of the gels.

2.4. *Crosslinking reaction with DEB [6]*

OS-ATPase (~5 mg/ml) was crosslinked at 50 mM triethanolamine (pH 8) with DEB at 0.5–1% (v/v). After 15–60 min incubation the reaction was stopped with excess methylamine. Subsequently the samples were dialyzed for 1 h at 20°C against 0.1 M SDS. Preparation for gel electrophoresis was similar to the procedure in section 2.3.

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2.5. *Crosslinking reaction with Cu-phenanthroline*

The reaction with *o*-phenanthroline and $CuSO_4$ was performed as follows: ~4 mg OS-ATPase protein was incubated in 50 mM triethanolamine buffer (pH 8) at 1.6 ml with 0.35 mM *o*-phenanthroline–0.17 mM $CuSO_4$ for 10–30 min at 20°C. Thereafter the reaction was terminated by making the solution 1.25 mM in EDTA, 16 mM in *N*-ethylmaleimide and 4% in SDS as in [11]. Subsequently the samples were prepared for electrophoresis by an analogous procedure to that in section 2.3.

2.6. *One- and two-dimensional slab gel electrophoresis*

Gradient polyacrylamide gel electrophoresis in SDS was performed in a 5–15% gel as in [2]. For the second dimension, the gels were overloaded with protein, to be able to detect crosslinked proteins. The gels from the first dimension were dissected into strips and washed with 10 mM sodium phosphate buffer, 0.1% SDS (pH 6). This buffer was changed after 7 min. Thereafter, the strips were put into the cleaving buffer containing 0.1% SDS, and 20 mM $NaIO_4$ (for TDGA and DEB crosslinks) for 30–40 min. For Cu-phenanthroline samples sodium periodate was replaced by mercaptoethanol in the cleaving buffer [11]. Subsequently the strips were dried with filter paper. Then they were bathed in the spacer gel solution for 15 min and afterwards polymerized into the second dimension gel. This consisted of 12% polyacrylamide.

2.7. *Sulfhydryl group titration*

Titration with dithionitrobenzoate (Nbs_2) [12] was done as follows: ~10–11 mg protein was incubated in 1600 μl 50 mM triethanolamine buffer (pH 8) at 20°C. DEB at up to 1% was added. After 60 min incubation the amount of SH-groups was titrated. For the titration with [ethyl-1-^{14}C]NEM (spec. act. 23.7 mCi/mmol, NEN Chemicals, Boston MA) 90 nmol/mg protein of a solution containing 99% [^{12}C]NEM and 1% [^{14}C]NEM was added to the OS-ATPase protein in 50 mM triethanolamine buffer (pH 8). NEM was added 1 min after the DEB (1%). Radioactivities before (100%) and after dialysis for 60 min (X%) was estimated in a Tricarb Scintillation counter.

2.8. *Estimation of ATPase activities*

ATPase activities were determined exactly as in [2] using the method in [10].

2.9. *Estimation of ATP–P_i exchange activities*

ATP–P_i exchange activities were measured by the method in [10] as modified [13].

2.10. *Substances*

All buffer substances were of the highest purity available. DEB was purchased from Merck (Darmstadt). TDGH was synthesized according to [5].

3. Results

Fig.1 shows the result after treating OS-ATPase with low concentration of TDGA (10 mM). From a crosslinked complex comigrating with proteins of M_r 55 000–65 000 (α- and β-subunits) two proteins are cleaved out by Na-periodate which can clearly be identified as the γ-subunit (34 kDa) and a 31-kDa protein.

When higher concentrations of the crosslinking reagent are used (40 mM) we obtain the result in fig.2. One set of stained protein spots appear below the 31-kDa protein band of apparent sizes 8-, 9-, 13- and 15-kDa. We conclude that a crosslinked complex of the former 3 proteins and a dimer of the latter were formed both resulting in complexes of M_r of ~30 000.

Another set of spots we observe below the 55–60-kDa proteins. Four weaker spots, two around

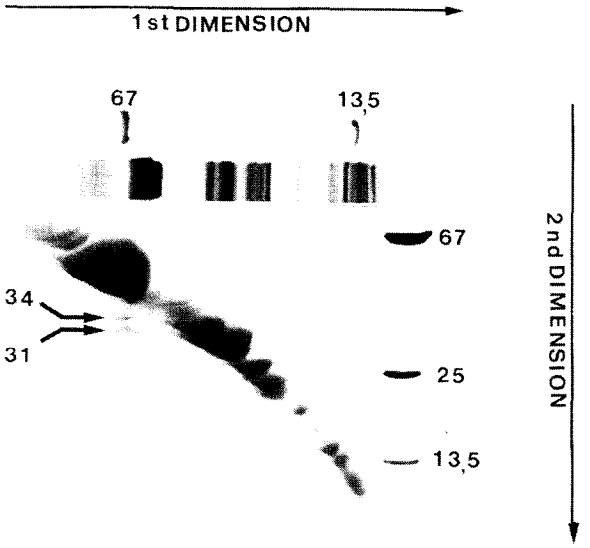

Fig.1. Crosslinking of OS-ATPase with 10 mM TDGA: Spots derived from 31- and 34-kDa bands are visible.

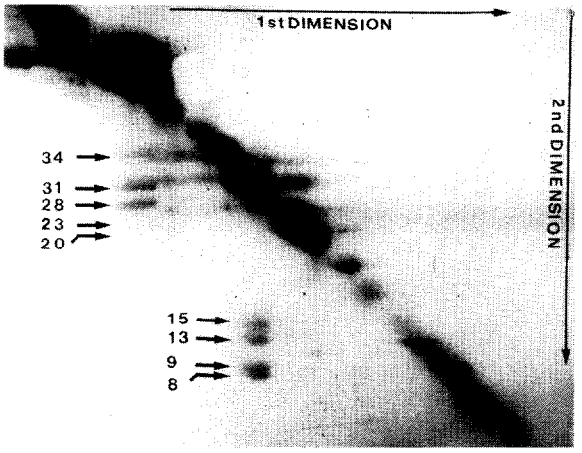

Fig.2. Crosslinking of OS-ATPase with 40 mM TDGA: Spots derived from proteins of 8-, 9- and 13-kDa, 15 kDa (dimer ?) and of 20 (23) kDa (faint spots) as well as of 34 kDa (faint) are visible. Strong spots are found at 31 and 28 kDa.

34-kDa and another couple at 20- and 23-kDa would add up to pairs of M_r 55 000–60 000. These spots also compare very well in their staining intensity. There are also two strong spots of M_r 28 000 and 31 000 which were cleaved out of the same complex on the diagonal indicating crosslinking between these subunits.

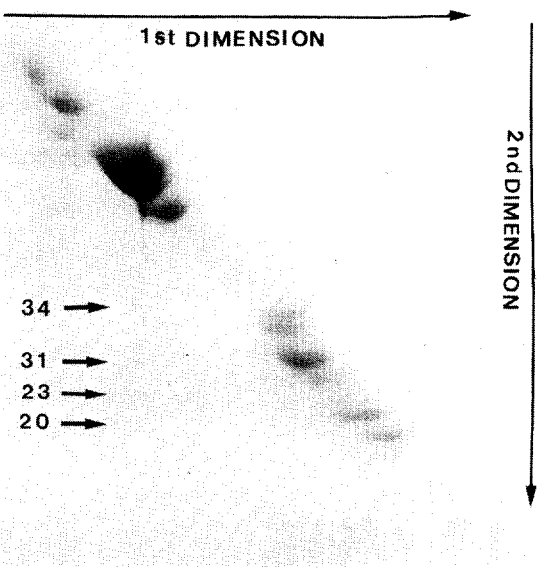

Fig.3. Crosslinking of OS-ATPase with Cu-phenanthroline: Spots derived from bands of 34 kDa and 20 kDa are visible, also of the band of 31 kDa and of a band at ~23 kDa.

Results obtained by crosslinking the ATPase with Cu-phenanthroline are shown in fig.3. Four stained spots, again below the 55–60-kDa region suggest crosslinking of the γ-subunit (34 kDa) with the 20-kDa

Table 1
ATPase and ATP–P exchange activities in presence of 1% DEB

ATPase activities (controls ~4–10 μmol P_i . min^{-1} . mg $protein^{-1}$)		ATP–P_i exchange activities (controls ~130 nmol . min^{-1} . mg $protein^{-1}$)
Incubation time (min)	% change compared with control	% change compared with control
0	0	
10	+4	
20	+26	
30	+35	
40	+63	−19.7 ± 7.75 (n = 8)

Table 2
Titration of sulfhydryl groups with 5,5′-dithiobis-(2-nitrobenzoic acid) (Nbs_2) or [ethyl-1-^{14}C]NEM after crosslinking reaction with DEB (1%)

Nbs_2	nmol/mg protein	[^{14}C]NEM	nmol/mg protein
Control	29.4	Control	35.45 (n = 2)
DEB	15.54 ± 0.95 (n = 4)	DEB	19.64 (n = 2)

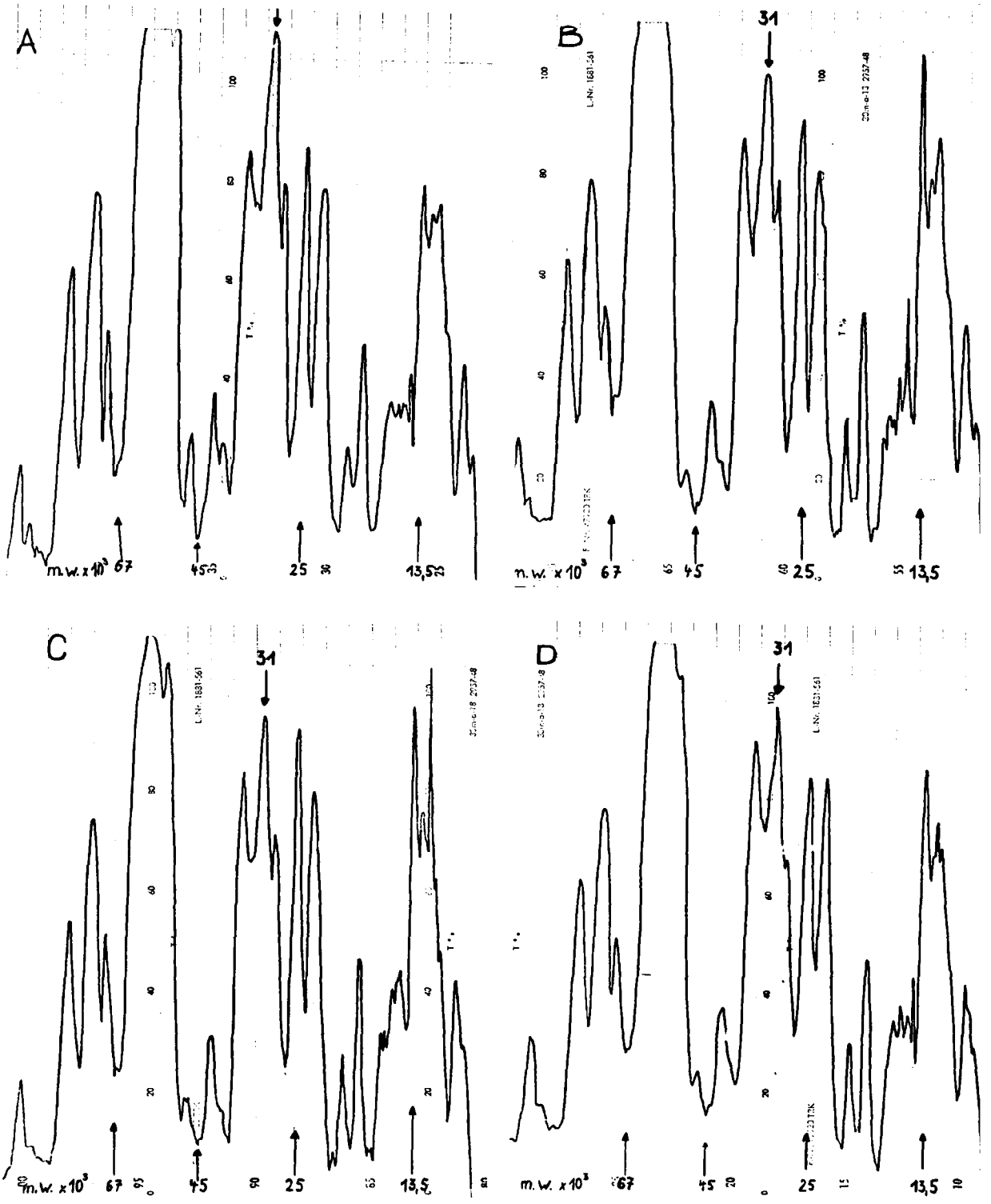

Fig.4. Crosslinking of OS-ATPase with 1% DEB. Decrease of staining intensity of 31-kDa band with time of incubation with DEB: (A) 15 min (similar to control); (B) 30 min; (C) 45 min; (D) 60 min. Note also changes of 20-kDa and in the low M_r range <15 000.

protein and of the 31-kDa protein with a second one of 23 kDa.

Fig.4 shows the result of reaction of DEB with the OS-ATPase as a function of time: The band at 31 kDa is decisively decreased with time of incubation, while ATPase activity is stabilized (table 1). A similar result was obtained, when DEB was varied from 0.5–1%.

The decrease of ATP–P_i exchange activity is also demonstrated in table 1. In table 2 we see the results of titration of SH-groups in OS-ATPase after reaction with DEB: The values are decreased by ~50%.

4. Discussion

Oligomycin sensitivity is obtained, when soluble ATPase (F_1) is bound to the membrane part (F_0) [14]. Different sites in F_0 are concerned with oligomysin sensitivity: The oligomycin binding site of M_r 8000–10 000, the OSCP, presumably in the 'stalk' part of the tripartite ATPase unit, M_r ~20 000, further the 31-kDa protein [1,2]. Knowledge of interrelations between these sites is important for an understanding of function of the OS-ATPase complex. Chemical crosslinking of the subunits of the ATPase is a method to learn about such interrelations.

One main point of our work is the observation that a connection between OSCP and the 31-kDa protein exists via the 34-kDa γ-subunit of F_1. The γ-subunit has been implicated in proton translocation as the proton gate [15]. This function should be shared in OS-ATPase (or transferred to) by the 31-kDa protein [2,3,13].

Crosslinking with DEB revealed a decrease in the 31-kDa band intensity, similarly as that observed with the uncoupler FCCP [3]. The amount of titratable SH-groups in presence of DEB is ~50% the control value. ATPase activity is, during prolonged incubation, stabilized by DEB while, concomitantly, ATP–P_i exchange activity is decreased.

Since there are no indications for M_r bands at >60 000 after reaction with DEB in the ATPase, it is anticipated that dimers of the 31-kDa protein are formed as was observed similarly with TDGA (not shown).

These are then contained in the bulk of α-, β-subunits of an M_r ~55 000–60 000. These dimers are most probably formed by DEB crosslinks involving sulfhydryl groups.

There also appears another crosslink between a 31-kDa and a 28-kDa protein. The identity of this latter protein, however, is uncertain.

Further evidence for a dimer of a 10-kDa (oligomycin, DCCD-binding site?) protein has been obtained. A 15-kDa protein also should be a dimer. Its identity is uncertain.

Crosslinks between low molecular mass of 8-, 9- and 13-kDa proteins are also of interest. A concomitant staining increase of low-molecular mass proteins (~7–15 kDa) and of a band of 30–34 kDa had been found to occur in rat liver mitochondrial membrane after addition of thiol reagent [16]. Further investigations should reveal a connection between the oligomycin (DCCD) binding site and the 20 000–30 000 M_r region.

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