2-MERCAPTOPROPIONYLGLYCINE RESTORES ACTIVITY OF OLIGOMYCIN-SENSITIVE ATPase TO CONTROL VALUES FOLLOWING TREATMENT WITH CARBONYLCYANIDE-*P*-TRIFLUOROMETHOXYPHENYLHYDRAZONE

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Received 3 September 1979

1. Introduction

We have shown [1] that the intensity of band 4 (mol. wt 31 000, nomenclature from [2]) of oligomycin-sensitive ATPase from beef heart mitochondria increases in the presence of thiol reagent, 2-mercaptopropionylglycine. Here we show that the intensity of this band (which is lacking in oligomycin-insensitive ATPase [3]) decreases in the presence of 0.2 μM of the uncoupler carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP). This decrease in band intensity is restored to the control level by subsequent treatment with 14 nmol/mg protein of 2-mercaptopropionylglycine. Furthermore, ATPase activity increased by FCCP is restored to the control level by 2-mercaptopropionylglycine.

Spin labeling of the mitochondrial ATPase with short and long chain maleimide labels was carried out. Sequential treatment with FCCP and 2-mercaptopropionylglycine revealed that mobile sulfhydryl regions of the ATPase molecule are stabilized by the thiol reagent against reduction induced by FCCP.

2. Materials and methods

2.1. *Isolation of beef heart mitochondria*

Beef heart mitochondria were isolated and submitochondrial particles prepared by the methods in [4,5].

2.2. *Preparation of oligomycin-sensitive ATPase complex*

Oligomycin-sensitive (OS) ATPase complex was prepared by the procedure in [2], thereby omitting the purification step by sucrose gradient centrifugation. The final pellets of OS ATPase were suspended in a buffer containing Tris–sulfate 10 mM, EDTA 0.5 mM, $MgSO_4$ 1.0 mM, sucrose 50 mM (pH 7.5) and stored at −70°C.

2.3. *Estimation of ATPase activities*

ATPase activity was estimated as in [2]. OS ATPase was diluted for the determinations with the same buffer as used for freezing the ATPase at −70°C, in addition to containing 10% glycerol. 10% glycerol was also added to the incubation buffer during incubation at 30°C. Asolectin (American Lecithin Co., Woodside, NY) was used for the activation step. In accordance with [2,3] the peak activities obtained were ~16.75 nmol $P_i \cdot mg^{-1} \cdot min^{-1}$ at 30°C.

First carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), if present, was added to the incubation buffer, and then the diluted OS ATPase suspension containing 2 μg protein/incubation vessel. 1 min later, 2-mercaptopropionylglycine, if present, was added.

2.4. *Incubation of OS ATPase for polyacrylamide gel electrophoresis*

OS ATPase at 8.5 mg protein/0.4 ml was incubated at 20°C in the same buffer in which it had been kept frozen at −70°C. 2-mercaptopropionylglycine, if present, was added 1 min after FCCP. After 30 min incubation the reaction mixture was dialysed for 60 min against water at 0–5°C. The dialysed fractions were subjected to electrophoresis as in [1].

2.5. *Spin labeling of OS ATPase with maleimide labels*

OS ATPase (100 μl) containing 2.5 mg protein was examined immediately after thawing. FCCP, if present, was added at 0.2 μM, 1 min thereafter, if present, 2-mercaptopropionylglycine was added. 1 min later 1 μl 5 mM solution of either spin label 100 or 114 (Syva) was added, mixed vigorously, and spectra were taken (200 scans) by means of a Bruker B-MN, 155-45 S 1 6 spectrometer at 22°C.

2.6. *Substances*

ATP was obtained from Sigma, München. 2-mercaptopropionylglycine was generously supplied by Santen Pharmaceut., Osaka. FCCP was purchased from Boehringer, Mannheim. Spin labels, 4-maleimido-2,2,6,6-tetramethyl-piperidinooxyl (no. 100) and 3-[(2-(maleimidoethoxy) ethyl) carbamoyl]-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (no. 114), were obtained from Syva, Palo Alto, CA.

All other chemicals were reagent grade of highest purity.

3. Results

The polyacrylamide gel electrophoretic patterns of oligomycin-sensitive ATPase in the presence of FCCP and 2-mercaptopropionylglycine (MPG) are shown in fig.1a–c. As follows from these results, the ratio of band 4/γ (with mol. wt 31 000 and 34 000, respectively) is strongly depressed in the presence of 0.2 μM FCCP (fig.1b). The addition of 2-mercaptopropionylglycine (14 nmol/mg protein) in the presence of FCCP

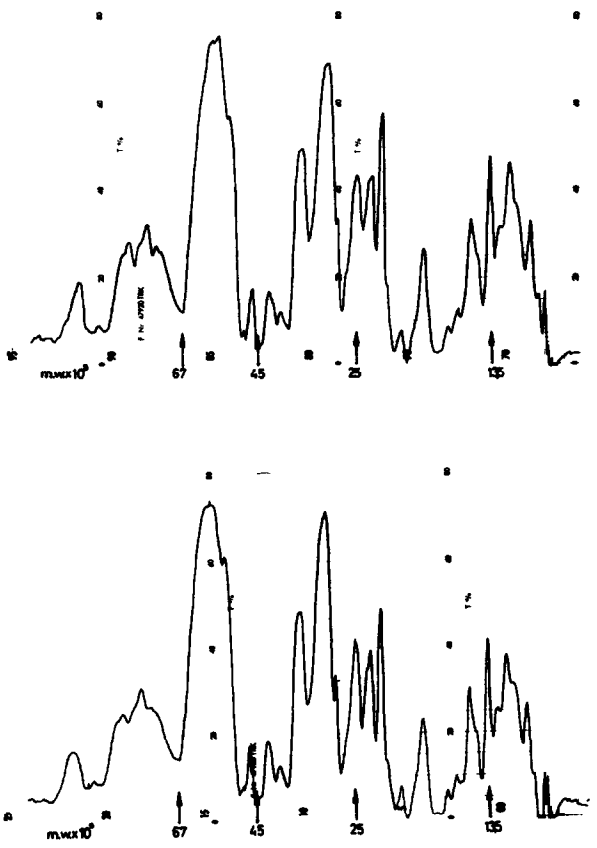
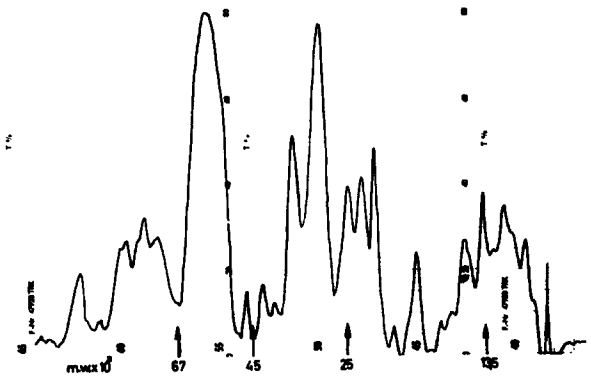


Fig.1. Electrophoresis of OS ATPase from beef heart mitochondria. Incubation conditions see section 2. (a) Control; (b) in presence of 0.2 μM FCCP; (c) in presence of 0.2 μM FCCP and, subsequently, 14 nmol/mg protein of 2-mercaptopropionylglycine.



leads almost to a total recovery of bands 4/γ ratio (fig.1c). A series of 17 experiments of this type has led to the results documented in table 1. It is clearly indicated that in these experiments the arithmetic mean of the controls can be restored by adding FCCP and 2-mercaptopropionylglycine. The statistical evaluation of the data was carried out by help of paired differences. It was observed that the differences between the controls and FCCP on the one hand, and between FCCP and FCCP/MPG on the other hand, are significant.

In a few cases it was not possible to measure the ratio of peak magnitudes between bands 4 and γ. Here we found that, in particular, band 4 was either

Table 1
Peak height ratios bands $4/\gamma$[a]

	Ratio	Mean paired difference ± SEM	n
Control	1.433	0.08 ± 0.012[c]	17
FCCP	1.355		17
FCCP + MPG[b]	1.432	0.107 ± 0.02[c]	17

[a] Nomenclature from [2]
[b] 2-Mercaptopropionylglycine
[c] Statistically significant $p < 0.01$

Experimental conditions similar to those in fig.1

broadened or split and, consequently, the peak height was reduced.

The results of ATPase activity determinations are shown in fig.2. In analogy to the experiments mentioned above, it was revealed that the ATPase activity stimulated by FCCP was brought to control values by the addition of MPG (58 nmol/mg protein).

Spin labeling data on OS ATPase give supporting evidence of the antagonistic effect of MPG versus FCCP (fig.3,4, table 2).

In fig.3, results with the short chain maleimide label no. 100 are presented. The control spectrum (fig.3a) is composite, revealing strongly as well as weakly immobilized parts, pointed out by arrows. Figure 3b gives results obtained after addition of 0.1 or 0.2 μM of the uncoupler FCCP. The weakly immobilized parts of the spectrum are rapidly reduced,

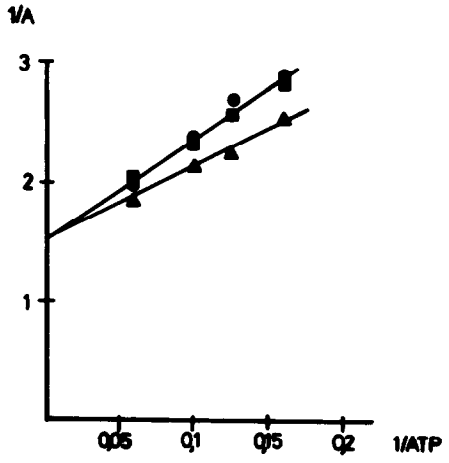


Fig.2. ATPase activity of OS ATPase in the presence of FCCP, restoration to control level by 2-mercaptopropionylglycine. (•) Control; (▲) in presence of 0.1 nM FCCP; (■) in presence of 0.1 nM of FCCP and, subsequently, of 58 nmol/mg protein of 2-mercaptopropionylglycine; 2 μg protein, 1 ml incubation vol. Further conditions see section 2. Each experimental point is based on 5 independent determinations.

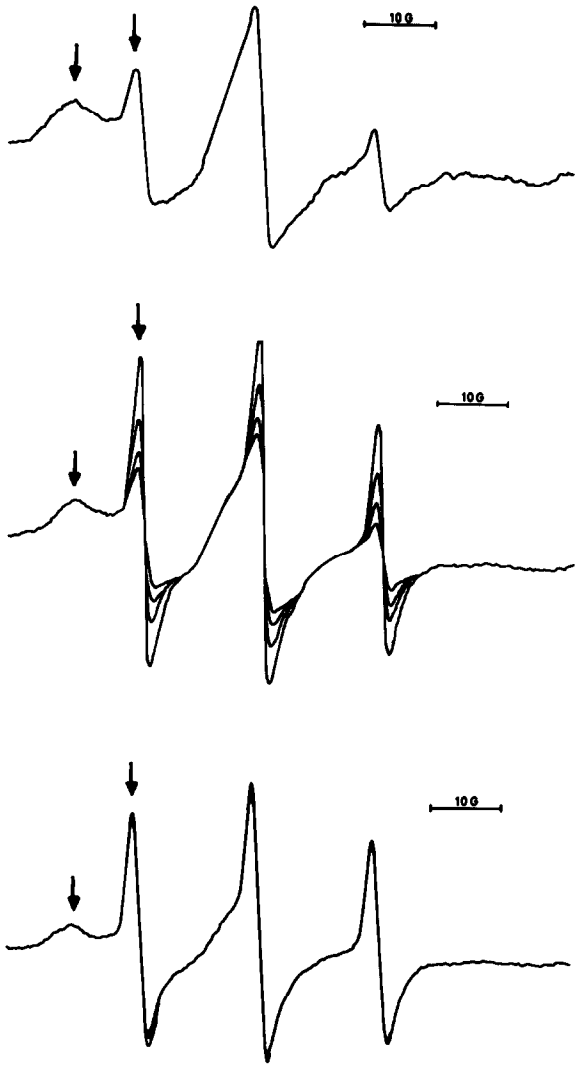
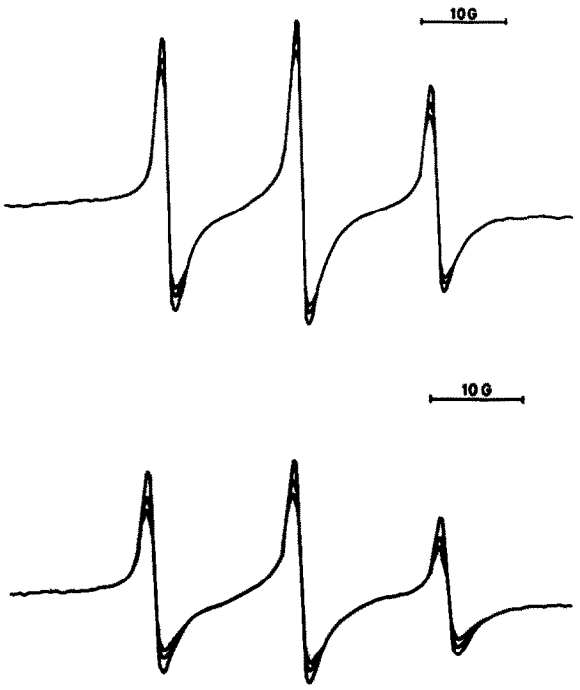


Fig.3. Spin labeling results on OS ATPase obtained with the short chain maleimide spin label (no. 100, Syva). (a) Control; (b) in presence of 0.2 μM FCCP; (c) In presence of 0.2 μM FCCP and, subsequently, of 4 nmol/mg protein of 2-mercaptopropionylglycine; Arrows indicate strongly and weakly immobilized parts of spectra respectively.

as can be found in spectral lines obtained by successive scans.

The result obtained after addition of 0.2 μM FCCP and, subsequently, of 4 nmol MPG/mg protein is shown in fig.3c. Here also the weakly immobilized part of the spectrum is accentuated; in contrast to fig.3b, however, the signal heights are stable during consecutive scans.

Results obtained with the long chain maleimide spin label are presented in fig.4a–c. The much more mobile type of spectrum does not exhibit strongly immobilized parts. Figure 4a shows an appreciable rate of reduction. This reduction of peak heights as found in consecutive scans is enhanced in the presence of 0.1 μM FCCP. Note the decrease in signal height at identical amplification of the ESR machine (fig.4b). In fig.4c, 1 min after FCCP, 16 nmol MPG/mg protein was added. The signal height is now stabilized. In table 2 ratios of h_0/h_{-1} (heights of midline signal over high magnetic field signal) are presented, which indicate an increase in mobility of the spin label molecules found in the presence of MPG. This result, however, may be also partly due to the decrease in rate of reduction.



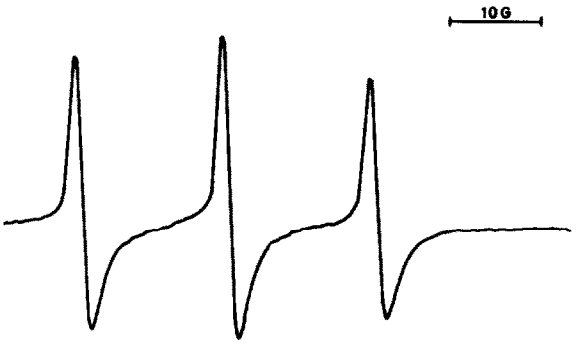


Fig.4. Spin labeling results on OS ATPase obtained with the long chain maleimide spin label (no. 114, Syva). (a) Control; (b) in presence of 0.2 μM FCCP; (c) in presence of 0.2 μM FCCP, and, subsequently, of 16 nmol/mg protein of 2-mercaptopropionylglycine.

4. Discussion

The influence of uncouplers of oxidative phosphorylation on the polar/apolar interface region of mitochondrial membranes is known [6,7]. Thus reactivity of membrane SH-groups, identified by the reduction rate of spin label molecules [8] is decreased by uncoupler [6].

As was found in the present study for the solubilized OS ATPase, mobile sulfhydryl regions of the molecule are stabilized by the thiol reagent MPG against the high reduction rate induced by FCCP.

Furthermore, the reactivity of primary amino groups is decreased by FCCP [7]. There are indications, moreover, that the activity of phospholipase A_2 in aged or uncoupled mitochondria is high [9]. Since the stimulation of phospholipase A_2 activity in erythrocyte membranes is dependent on an increase

Table 2
Ratios h_0/h_{-1}[a] with spin label 114 in OS ATPase

Control	FCCP (0.2 μM)	FCCP + MPG (nmol/mg prot.)	
1.45	1.58	1.49	4
		1.38	8
		1.25	16
		1.25	24

[a] Line heights of first scan were measured

in the number of disulfide bridges [10], one may suppose that analogously FCCP increases the number of disulfide bridges in mitochondria. No proof, however, for such mechanism occurring in mitochondria is presently available.

It is interesting that Weiss and McCarty [11] using a bifunctional maleimide in their studies on chloroplast ATPase were able to obtain crosslinkings in the γ subunit. This crosslinking between functionally different SH-groups resulted in an uncoupling and higher proton permeability of the thylakoid membrane.

As already discussed [1], MPG may facilitate an SH–S–S interchange reaction in oligomycin-sensitive ATPase of beef heart. The effects of FCCP and MPG on the ATPase activity as well as on the ratios between band 4 and the γ subunit here may also due to SH–S–S interchange reaction.

Acknowledgements

We thank Drs Gerhard Burckhardt and Rolf Mehlhorn for valuable discussion of the paper. Thanks are also due to Dr Bernd Lämmel for providing the ESR facilities.

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