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OLIGOMYCIN SENSITIVITY OF MITOCHONDRIAL SULFHYDRYL GROUPS

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SUMMARY

Dithionitrobenzoate has been used to titrate sulfhydryl groups of rat liver mitochondria in glutamate buffer, pH 7.4.

Reaction with oligomycin and different SH reagents preceded the SH titration. Under these conditions it was found that 2-mercaptopropionylglycine and *N*-ethylmaleimide reacted in an oligomycin-sensitive manner, so that the control values (in the absence of SH reagent) were obtained.

Similar concentrations of mersalyl and of *N*-(*N*-acetyl-4-sulfamoylphenyl) maleimide, in the presence of oligomycin, enhanced reactivity toward Nbs_2.

The concentration range of oligomycin-sensitive SH groups was thus defined between approx. 5 and 9 nmol reagent/mg mitochondrial protein.

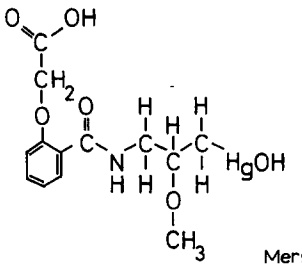
In this way, a differentiation between SH groups, which are implicated in phosphate transport and those, which react in an oligomycin-sensitive manner, and which are probably connected with the coupling mechanism, was achieved.

INTRODUCTION

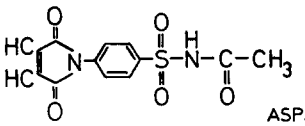
One main problem still unresolved with regard to mitochondrial membrane transport and phosphorylation concerns a differentiation in between transport-sensitive and oligomycin-sensitive sulfhydryl groups.

There are different sulfhydryl reagents of varying specificities, which may be suitable for reaction at the membrane level of organisms or organelles like mitochondria. Mersalyl [1, 2], *N*-(*N*-acetyl-4-sulfamoylphenyl)maleimide (ASPM) [3], as well as 6,6-dithionicotinic acid [4] have been used in the case of the phosphate carrier, other reagents have been employed by other workers in the field of mitochondrial membranes [5–9].

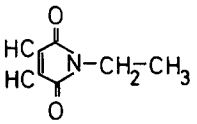
Since it was found that a surplus of about 30 % of sulfhydryl groups become reactive at the mitochondrial membrane in the presence of ADP [7], or ADP-P_i [6], and furthermore, that this increase in reactivity is oligomycin sensitive [6, 8], we have been interested in the search for a reagent, specific enough as to allow



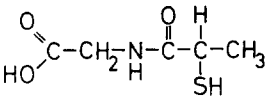
Mersalyl



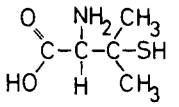
ASPM



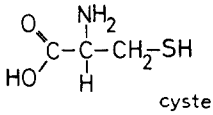
MalNEt



2-Mercapto-propionyglycine



penicillamine



cysteine

characterization of these oligomycin-sensitive SH groups. Besides, this reagent should, if possible, not seriously interfere with energy metabolism.

The 2-mercaptopropionylglycine which has been used in treatment of chronic hepatitis fulfills the second condition formulated above. There is ample evidence that oligomycin-sensitive mitochondrial sulfhydryl groups do react with 2-mercaptopropionylglycine [10]. More important, electron microscopic studies have revealed that this reagent is able to enhance the effect of ADP in creating condensed configuration of rat liver mitochondria (Zimmer, G. and Schneider, M., unpublished).

This reagent (some preliminary studies with this reagent have been reported previously [11, 12]) was consequently tried along with the already introduced β-mercaptovaline (penicillamine). These two sulfhydryl reagents have in common that the SH group is sterically shielded by one or two methyl groups.

These reagents have been compared with others like mersalyl, *N*-ethylmaleimide (MalNEt), ASPM and cysteine.

MATERIALS AND METHODS

Rat liver mitochondria from animals of the Wistar strain were prepared in a conventional manner using 0.25 M sucrose as the preparation medium. Mitochondria from three rats were collected in a volume of 1.5 ml containing about 60–100 mg protein/ml.

5,5′-Dithiobis-(2-nitrobenzoic acid) (Nbs_2), Ellman's reagent [13], dithioerythritol, D-penicillamine and mersalyl were obtained from Serva, Heidelberg, adenosine diphosphate and oligomycin from Sigma. *N*-Ethylmaleimide (MalNEt) was purchased from Schuchardt, München, and sublimated before use. *N*-(*N*-acetyl-4-sulfamoylphenyl)maleimide (ASPM) was a gift from Professor Gerhard Pfleiderer, Stuttgart, 2-mercaptopropionylglycine was generously supplied by Santen Pharmaceutical Co., Ltd., Osaka, Japan. Rotenone was purchased from Ega-Chemie,

Steinheim/Heidenheim, L-cysteine was obtained from Merck All other reagents were of analytical grade.

SH titrations. Titrations of mitochondrial sulfhydryl groups were carried out in glutamic acid buffer 0.01 M Tris, 0.015 M with HCl, pH 7.4 [6].

(a) Control experiments in the absence of added oligomycin: 25 μl of mitochondrial suspension, containing about 1.5–2.5 mg of protein were added to 3 ml of the buffer at 27 °C. 2 min later Nbs_2 was added in an amount sufficient to give a 10–20-fold molar excess of reagent over the expected SH groups. It was measured against a control without Nbs_2 set to a 100 % transmission.

(b) Experiments with addition of SH compounds or thiol blocking reagents in the absence of added oligomycin· 25 μl of mitochondrial suspension, containing about 1.5–2 5 mg of protein were added to 3 ml of the buffer at 27 °C. 1 min later the SH compound or thiol blocking reagent was added, and, after a further min, Nbs_2 was added as above in excess.

(c) Experiments carried out in the presence of oligomycin: The experimental procedure was exactly as was described under a or b with the exception that oligomycin (dissolved with absolute ethanol) was added to the buffer before addition of mitochondria (at least 1 μg/mg protein). Measurements were carried out with a Zeiss PMQ II spectrophotometer at 412 nm.

It was presumed, that in the concentration range of SH reagent used, binding to mitochondrial sites occurred quantitatively. Moreover, the SH reagents, when titrated with Nbs_2 in the absence of mitochondria, did not give a stoichiometric reaction, since the concentration range used is near the limits of sensitivity to Nbs_2. Therefore, no attempt was made, to remove free SH reagent before titration with Nbs_2.

Permeability of the mitochondrial membrane to the reagents used is presumed to be as follows· MalNEt, permeant [9, 14, 15]; 2-mercaptopropionylglycine, probably permeant; D-penicillamine, probably permeant; cysteine, permeant; mersalyl, not permeant [14, 15], ASPM, not permeant [16].

Phosphate transport. Measurements of phosphate transport were executed as described by Fonyo et al. [2] with the following variations: Dithioerythritol was used instead of 2-mercaptoethanol. The excess of reagent over mersalyl was about 4–5-fold on a molar basis The temperature was 15 °C instead of 10 °C. Oligomycin (at least 1 μg/mg protein) was added initially to the incubation medium.

RESULTS

All titration results are collected in Table I. The control values in the absence of added SH reagent are shown not to be different in the presence or absence of added oligomycin.

Mean concentrations of the reagents, used, are indicated. With *N*-ethylmaleimide we find for a concentration range of 7–8 nmol/mg protein values, that are well below those of the controls by about 30 %. In comparison, in the presence of oligomycin, we observe a value that is nearly identical to the one of the controls This behaviour is, however, strictly concentration dependent At 9.5–12 nmol, there is no longer an effect of oligomycin to be observed.

Considering the values in the presence of 2-mercaptopropionylglycine, we find that in between concentrations of 1 5 and 4 nmol/mg protein the SH-titrated do not

TABLE I

TITRATION OF MITOCHONDRIAL SH GROUPS BY AN EXCESS OF Nbs_2

The experiments were carried out as described in Materials and Methods The values shown were obtained after a reaction time of 15 min with Nbs_2

Addition	Mean concentration (nmol reagent/mg protein)	SH titrated (nmol/mg protein)	
		−oligomycin	+oligomycin
None		32 1 ±2 3 (21)	32 6±3 8 (8)
MalNEt	7–8	22 75±1.5 (4)	32.6±1 7 (5)
	9 5–12	17 0 (2)	18 8±6.1 (4)
2-Mercapto-propionyl-glycine	1.5–2	30 9 ±0 6 (3)	32 6±1.3 (5)
	4	32 0 ±4 0 (3)	34 5±2 0 (5)
	5–5 5	35 0 ±5.5 (4)	34 3±0 9 (4)
	7	41 5 ±5 1 (4)	31 7±2 1 (7)
	9 5–10	41.8 ±4 2 (4)	40.0±4 6 (3)
Mersalyl	3	22.1 ±5 1 (7)	23 3±4 6 (8)
	6	23 2 ±2 1 (7)	28 1±1 4 (6)
	9	23 8 ±3 8 (7)	21 8±2 7 (6)
ASPM	7–8	20 5 ±2 6 (4)	27 0±1.4 (4)
Penicillamine	6 5–7	24 8 ±4 9 (6)	33 5±0 6 (3)
Cysteine	6 5–7	52 8 ±5 7 (4)	36.6±1.5 (3)
	10	51 8 ±4 (6)	51.0±5 6 (5)

much differ. It is of interest, however, that from about 5–7 nmol onward there is a distinct increase of the titration values in the absence of added oligomycin. If oligomycin is present, at 7 nmol of the reagent, a value near the controls is obtained. Further increasing the concentration of this reagent brings about increases of SH titrated irrespective the presence or absence of added oligomycin. This is comparable to what is found with *N*-ethylmaleimide titration.

Using mersalyl, we find, at approximately 3 nmol/mg protein that titration values of about a similar range compared to MalNEt are obtained. This is the case both in the absence or presence of added oligomycin. A similar result is observed for a concentration of about 9 nmol/mg protein. In contrast, at approximately 6 nmol/mg protein, we discern that in the presence of oligomycin, the titration values rise by about 20 %. A similar result is obtained using ASPM at 7–8 nmol/mg protein (about 30 % increase).

The difference in titration behaviour between the SH compound 2-mercaptopropionylglycine and the thiol blocking reagent mersalyl is clearly brought out in Fig. 1: While there is an unmasking of SH groups by the former reagent, indicated by the higher titration values compared to the control, the latter reagent on the contrary either masks sulfhydryl groups, or, more probably, reacts with SH groups that are otherwise reactive to Nbs_2.

With penicillamine, again, the values at the concentration range of 6.5–7 nmol/mg protein are comparable to those obtained with mersalyl and ASPM (oligomycin absent), while in the presence of oligomycin the control values are nearly obtained (Table I).

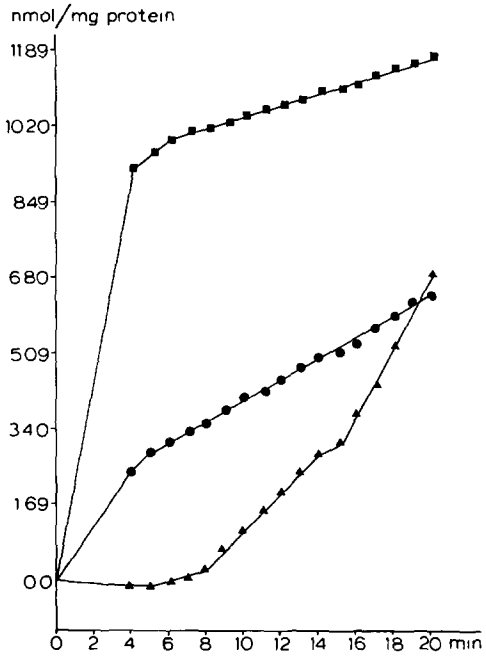


Fig. 1. Titration of mitochondrial SH groups by limited amounts of Nbs_2 ●, control, ■, 2-mercaptopropionylglycine (37 μM), ▲, mersalyl (43 μM). 200 μl of mitochondrial suspension (about 14 mg of protein) was added to 3 ml Tris/glutamate buffer After preincubation for 1 min (27 °C) SH reagent was added at the concentration as indicated. 1 min later 0 25 μmol of Ellman's reagent was added; this is at least 2-fold molar excess of mitochondrial sulfhydryls over Nbs_2.

Finally with cysteine, at 6.5–7 nmol/mg protein there is an effect of oligomycin to be observed since the values are decreased by about 30 % compared to those obtained in the absence of oligomycin. This effect is abolished at higher concentration of the reagent.

DISCUSSION

The range of values of SH groups, as exhibited in Table I, is lower in comparison to that found by Sabadie-Pialoux and Gautheron [6] and approaches that estimated by Chude and Boyer [17] in liver and heart mitochondria. Since specificity of the results apparently is decreased in the course of time (see Fig. 1) the reaction time with Nbs_2 was confined to 15 min (see Table I). The main concern of this work, however, was not to estimate quantitatively total SH groups, but to differentiate between titrations at varying (metabolic) state of the mitochondria.

For the oligomycin-sensitive range of 5–9 nmol reagent/mg protein, we observe an unmasking of sulfhydryl groups by permeable SH compounds, if oligomycin is absent. In the presence of oligomycin, an unmasking of SH groups is brought about by non-permeable reagents (mersalyl, ASPM).

This implies some structural interdependence between oligomycin and/or nonpolar SH groups and polar sulfhydryl regions of the mitochondrial membrane.

The latter polar sulfhydryl regions of the membrane, however, cannot be classified as phosphate transport area, since phosphate transport inhibition due to mersalyl under the conditions of Fonyo et al. [2] was not affected by oligomycin (not shown). On the other hand, phosphate transport is only inhibited by ASPM at about 11 nmol/mg protein of reagent [3].

Oligomycin thus appears to decisively influence the availability or non-availability of SH groups at certain membrane regions, which are unrelated to phosphate transport.

The present paper is, in this respect, complementary to recent observations of Le Quoc et al. [18]. These authors described differences in accessibility or reactivity of mitochondrial thiol groups during different energetic state. It could not be ruled out, however, that part of the unmasked thiols were implicated in phosphate transport.

Possible analogies between SH groups, unmasked by ADP, and a similar process brought about by 2-mercaptopropionylglycine are of interest. It is not yet clear, whether SH groups, trapped by *N*-ethylmaleimide which are unmasked by small concentrations of ADP [19] belong to the ADP-ATP carrier or to proteins in close proximity to the transport region [20]. It should be noted, that there also seems to exist a close relationship between the mitochondrial energy coupling system (ATPase) and the region of the ADP-ATP translocation [20].

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