Characterization of sulphydryl groups of the mitochondrial phosphate translocator by a maleimide spin label

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A maleimide spin label strongly inhibits the phosphate/H^+ symporter of rat liver mitochondria. While inducing half-maximal inhibition of transport, the spin label reacts preferentially with the SH groups of the carrier, which are at least of two types. One type of SH group is localized close to the surface of the membrane and its environment does not significantly influence the mobility of the probe. The second type of SH group is buried in the membrane, is not accessible to ascorbate or chromium oxalate and its environment greatly restricts the motion of the probe.

Phosphate transport *Mitochondria* *Sulphydryl group* *Maleimide spin label*

1. INTRODUCTION

The electroneutral phosphate/proton symporter of the inner mitochondrial membrane is the major system for the supply of phosphate for oxidative phosphorylation [1]. The transport depends on the transmembrane ΔpH [2] and the carrier protein contains free SH groups which are required for its function [3,4]. The curve of inhibition of the carrier activity by different thiol reagents is sigmoidal [1,5]. This observation, together with the protective effect by non-inhibitory concentrations of mersalyl (a reversible ligand of SH groups) against the irreversible inhibition by NEM, has led to the suggestion that the phosphate carrier has two types of SH groups/unit [5–7].

Since SH groups currently represent the only well-defined functional groups of the carrier protein, further insight into the molecular mechanism of phosphate transport might be gained by investigating their physico-chemical properties in more detail.

Abbreviations: BSA, bovine serum albumin; NEM, *N*-ethylmaleimide; MSL, maleimide spin label; SDS, sodium dodecylsulfate

The attempt of this study was to use a paramagnetic analog of NEM (maleimide spin label) to characterize some properties of the phosphate carrier SH groups. It was found that MSL strongly inhibits phosphate transport of intact rat liver mitochondria. A predominant binding of MSL to phosphate carrier was detected and two types of reacting SH groups could be distinguished. They differed in their surrounding environment, accessibility and the depth at which they are localized in the membrane.

2. MATERIALS AND METHODS

N-[^{14}C]Ethylmaleimide (10 mCi/mmol) was obtained from New England Nuclear; Triton X-100 from Merck; Celite 535 from Roth; hydroxylapatite (Bio-Gel HTP) from Bio Rad; NEM and Mersalyl from Serva; maleimide spin label (*N*-[3-(2,2,5,5-tetramethyl-1-pyrrolidinyloxyl)]-maleimide) from Syva (Palo Alto CA). The length of MSL (spacer $-CH_2-$) is 7.9 Å [8].

2.1. *Preparation of mitochondria*

Rat liver mitochondria were isolated in 0.25 M

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sucrose, 10 mM Tris–HCl, 1 mM EDTA (pH 7.4) (STE medium). When indicated, mitochondria were preincubated with 15 nmol mersalyl/mg protein for 1 min at 0°C followed by the addition of 2 mM NEM. After further 2 min the reaction was stopped by 5 mM cysteine and the mitochondria were washed (3 times) with STE medium containing 0.5 mM cysteine (in the first wash). Incubation of mitochondria with MSL was stopped by 2 mM cysteine followed (for ESR measurements) by centrifugation in STE medium supplemented with 0.2% BSA (to remove the non-specifically bound MSL) and 5 mM ferricyanide (to protect the nitroxide group against irreversible oxidation). The mitochondria were washed repeatedly until the supernatant was free of the spin label (as controlled by ESR spectra of concentrated supernatants). Labeling of mitochondria with [^{14}C]NEM (14 nmol/mg protein, 2 min, 0°C) was performed as in [9].

2.2. *Isolation of phosphate carrier*

[^{14}C]NEM-labeled mitochondria were solubilized for 30 min by 4% Triton X-100, 50 mM KCl, 10 mM KPO_4, 1 mM EDTA, 1 mM dithiothreitol, 10 mM Tris–HCl (pH 7.4) and the unsolubilized material was sedimented (100000 × *g*, 45 min). Chromatography on hydroxylapatite and celite was performed in Pasteur pipettes as in [10].

2.3. *ESR measurements*

For ESR measurements an aliquot of the mitochondrial pellet was introduced into a glass capillary and the spectra were recorded at room temperature in a Jeol JES PE-3X spectrometer. Instrument settings were as follows: field set, 3268 ± 100 G (diphenylpicrylhydrazyl EPR marker); frequency and intensity X-band, 9.22 GHz and 20 mW, respectively; field modulation amplitude, 1.6 G; time constant 1 s; chart speed, 16 min/360 mm.

2.4. *Analytical methods*

Phosphate transport was measured by passive swelling as in [11]. SDS-gradient polyacrylamide slab gel electrophoresis of acetone-precipitated samples was performed as in [12]. Radioactivity in gels was detected by fluorography [13]. Protein concentration was determined as in [10].

3. RESULTS AND DISCUSSION

Preincubation of rat liver mitochondria with MSL for 2 min (0°C, terminated by 2 mM cysteine) results in a pronounced inhibition of phosphate transport (fig.1). To achieve a 50% inhibition of ammonium phosphate-induced swelling, ~24 nmol/mg protein of MSL were required (only 1.9-times more than the amount required for NEM).

As shown in [10], a short-term incubation of mitochondria with a low, inhibitory concentration of [^{14}C]NEM results in a specific labeling of the phospate translocator due to the high reactivity of its SH groups. To test further which of the mitochondrial SH groups reacted with MSL, mitochondria were incubated with a 50% inhibitory concentration of MSL as above and after washing out the cysteine and the unbound MSL

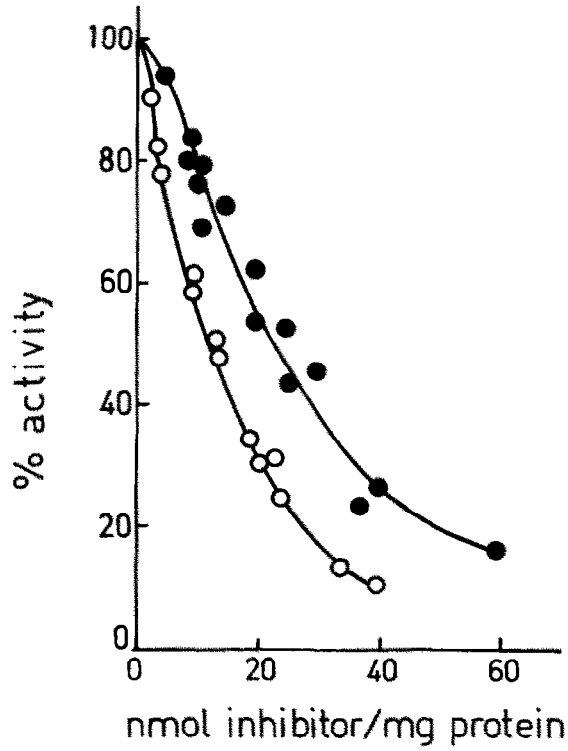

Fig.1. The inhibition of phosphate transport by NEM and MSL. Mitochondria (5 mg protein/ml) were incubated for 2 min with NEM (—○—) or MSL (—●—). Reaction was quenched by 2 mM cysteine and aliquots were transferred into 0.1 M ammonium phosphate (pH 7.2). A_{540} was measured.

they were labeled with [^{14}C]NEM. In control, non-preincubated mitochondria, the most of the bound radioactivity was associated with proteins of M_r 33000 and 31000 (fig.2), which had an equal mobility as the main radioactive components of the isolated phosphate carrier prepared from the control [^{14}C]NEM labeled mitochondria [10]. The preincubation of mitochondria with MSL (25 nmol/mg protein; i.e., 50% inhibitory concentration) resulted in a 40% decrease (mean value of 4 expt) of the total bound radioactivity. This was mostly represented by reduction of radioactivity associated with proteins of M_r 33000 and 31000 (fig.2). Only a minor part of the total decrease of radioactivity was due to the change in labeling of a low-M_r protein (14500). The identity of this component is not known but an SH protein of similar size was described to be one of the subunits of F_o [14].

Therefore, both the data on inhibition and on [^{14}C]NEM-labeling of the phosphate carrier indicate that the nitroxide group does not alter

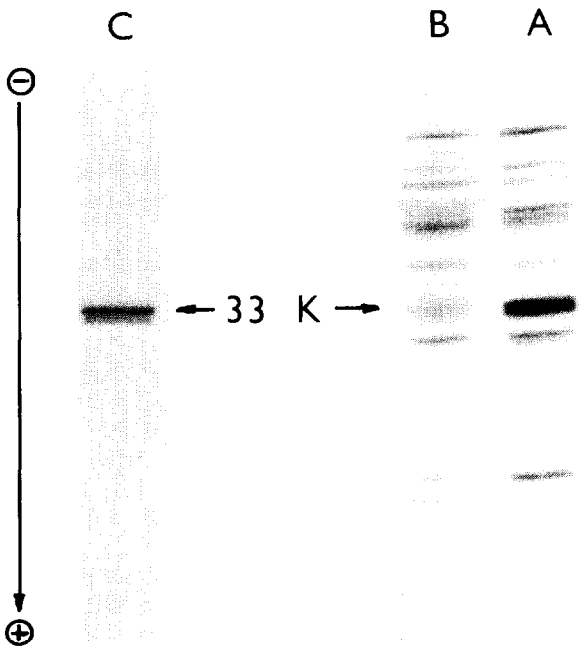

Fig.2. The effect of MSL on [^{14}C]NEM-labeling of mitochondria – electrophoretic pattern of labeled proteins: (A) control [^{14}C]NEM (14 nmol/mg protein) labeled mitochondria; (B) MSL (25 nmol/mg protein) preincubated mitochondria; (C) phosphate carrier isolated from (A).

significantly the specificity of interaction of maleimide with the phosphate carrier in intact mitochondria and, under the conditions used, the preferential binding of MSL to SH groups of the carrier is to be expected. Thus, in further experiments mitochondria were incubated with 25 nmol MSL/mg protein and the unreacted MSL was removed by extensive washing in the presence of BSA (section 2). The ESR spectrum observed (fig.3B) can be regarded as a superimposition of spectra from 2 kinds of bound spin labels, the molecular motions of which are restricted to different degrees. As compared with the ESR spectrum of MSL in buffer (fig.3A) (hyperfine splitting 15.3 ± 0.2 G of the 3 lines spectrum of about equal amplitude) a new component in the low field region occurred (fig.3B). This component apparently corresponds to the strongly immobilized signal with respect to the more mobile component representing a large fraction of the signal. The weakly immobilized signal might reflect the spin probe bound to an external site (water phase), whereas the strongly immobilized signal reflects the spin label restricted in its local motion [15].

On the basis of the above [^{14}C]NEM experiments, MSL, under the same conditions, should react preferentially with the SH groups of the phosphate carrier but not necessarily only with them. Therefore, to increase further the specificity of the MSL–phosphate carrier interaction, the SH groups of the carrier were protected by 15 nmol mersalyl/mg protein [16] and the remaining SH groups were blocked by NEM (section 2). After removing the bound mersalyl, MSL was added as above. As a result of this more selective labeling of the phosphate translocator, P_i transport was inhibited at somewhat lower concentration (20 nmol/mg protein MSL were required for 50% inhibition) and the shape and amplitude of the ESR spectrum changed (fig.3C). The strongly immobilized component increased significantly as compared to the weakly immobilized signal suggesting that a higher relative concentration of MSL or rather a decrease in competing bulk SH groups was needed to reach some of the SH groups of the carrier, the environment of which greatly restricts the mobility of the probe. Both types of signal undoubtedly represent MSL which has reacted with SH groups (and not free spin label), since preincubation of mitochondria with NEM, followed

by cysteine and washing, resulted in a drastic reduction of all lines amplitude (fig.3D).

The composite ESR spectrum of MSL bound to mersalyl-protected mitochondria may be interpreted to indicate a dual localization of the phosphate-carrier sulphydryl groups. To test this possibility, membrane-impermeable nitroxide group-attacking agents (ascorbate and chromium oxalate) were used, since only the signal of externally localized spin probe is either reduced [17] or broadened [18] by these agents. The weakly immobilized component of the spectrum gradually decreased after the addition of ascorbate (fig.4A), whereas the strongly immobilized component remained nearly unchanged. Similarly, chromium oxalate (fig.4B), although having a different mode of action (signal broadening), affected preferentially the weakly immobilized component of the spectrum, indicating that the strongly immobilized probe and the corresponding SH groups are localized more deeply in the membrane. In other experiments (not shown) MSL with different length of the chain linking the nitroxide group to maleimide were tested at a concentration giving ~50% inhibition of phosphate transport in mersalyl-protected mitochondria. It was found that with MSL's 6.8 Å long (no spacer) and 11.6 Å long (spacer $-CO-NHCH_2CH_2-$) [8] both the strongly and the weakly immobilized components of the spectrum were present in good agreement with fig.3C (MSL, 7.9 Å). With MSL 15.3 Å long (spacer $-CO-NHCH_2CH_2OCH_2CH_2-$) [8], however, the ESR spectrum shows only the weakly immobilized component. Assuming equal perpendicular location of the various MSL analogs in the membrane, this would suggest that the distance of the deep sites from the membrane surface might be < 15 Å.

The previous information about the functional SH groups of the phosphate carrier was mainly

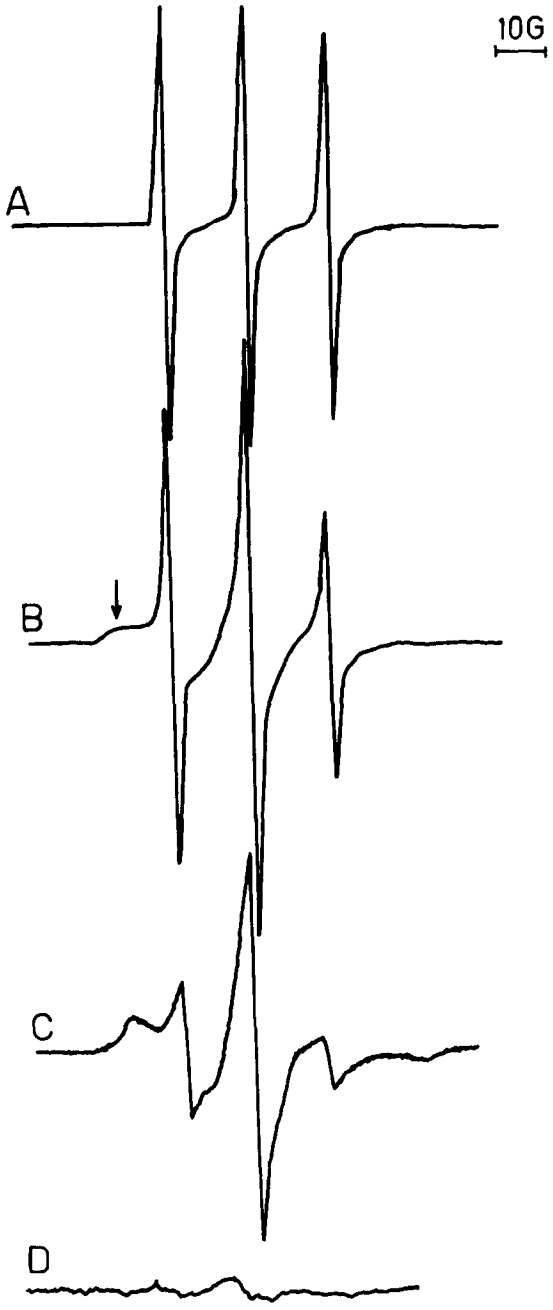

Fig.3. The ESR spectrum of MSL bound to mitochondria: (A) 1 μM MSL in STE buffer; (B) MSL bound to (control) mitochondria; 25 nmol MSL were added/mg mitochondrial protein and after 2 min reaction was stopped by 2 mM cysteine. Mitochondria were then extensively washed in the presence of BSA; (C) MSL bound to mersalyl–NEM–cysteine-pretreated mitochondria; mitochondria were first treated with mersalyl (15 nmol/mg protein) followed by NEM (2 mM) and cysteine (5 mM). After washing, mitochondria were treated with MSL (25 nmol/mg protein) as in (B); (D) MSL bound to NEM–cysteine-pretreated mitochondria; mitochondria were first treated with NEM (2 mM), then with cysteine (5 mM). After washing, mitochondria were treated with MSL (25 nmol/mg protein) as in (B). Amplification: (B) 1×10^2, (C,D) 1×10^3. For other details see section 2.

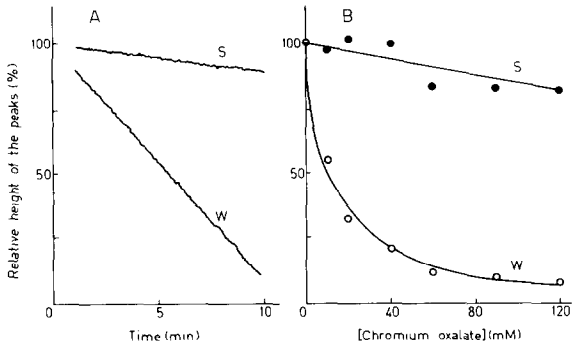

Fig.4. The effect of ascorbate and chromium oxalate on the weakly and the strongly immobilized components of the ESR spectrum from mersalyl protected mitochondria labeled with MSL. Mitochondria were protected with mersalyl and labeled with MSL as in fig.3C. Ascorbate (0.5 mM) in (A) and increasing concentrations of chromium oxalate in (B) were added at time zero. The strongly immobilized component (S) and the weakly immobilized component (W) were measured at 3256 G and at 3267 G, respectively, as a function of time in (A) and after 2 min in (B).

achieved by testing the inhibitory effects of various sulphydryl reagents of different specificity and permeability [5–7,16,19,20]. The results presented here indicate that paramagnetic analogs of NEM represent a suitable tool to investigate further the physico-chemical properties of the free, functionally important SH groups of the phosphate carrier. On the basis of measurements performed with intact mitochondria it is concluded that:

(i) MSL strongly inhibits phosphate transport via the NEM-sensitive phosphate/proton symporter;

(ii) When MSL induces a half-maximal inhibition of transport, it reacts preferentially with SH groups of the phosphate carrier and the specificity of the interaction can be further increased by blocking the bulk SH groups of the membrane;

(iii) MSL is then bound to two types of SH groups: One type of SH group is easily accessible, being localized at the surface of the membrane. Its environment does not significantly influence the mobility of the probe. On the contrary, the second type of SH group is less accessible and its environment greatly restricts the motion of the probe.

Since the difference in the mobility and the accessibility of bound MSL is so pronounced, these data support the view that the phosphate carrier contains at least two non-equivalent SH groups. However, it cannot be decided yet whether these are re-orienting or stable positions.

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