Phosphorylation of mitochondrial membrane proteins: effect of the surface potential on monoamine oxidase

Konrad S. Famulski, Maciej J. Nałęcz and Lech Wojtczak

Department of Cellular Biochemistry, Nencki Institute of Experimental Biology, Pasteura 3, 02-093 Warsaw, Poland

Received 19 April 1983; revision received 10 May 1983

Proteins of mitochondrial membranes become phosphorylated when liver mitochondria are incubated with ATP in the presence of Mg^{2+}. This is accompanied by an increase of the negative surface potential of mitochondrial membranes, as calculated from the dissociation constant of fluorescent probes, 8-anilino-1-naphthalene sulphonate and ethidium bromide, and by a decrease of the apparent K_m-value of mitochondrial monoamine oxidase. Protein phosphorylation, the increase of the negative surface potential and the decrease of apparent K_m of monoamine oxidase are greatly potentiated by cytoplasmic protein kinases in the presence of cyclic AMP.

Protein phosphorylation *Surface potential* *Monoamine oxidase* *Mitochondria* *Membrane protein* *Cyclic AMP*

1. INTRODUCTION

It has been shown [1–3] that the surface potential of natural and reconstituted membranes affects apparent K_m-values of membrane-bound enzymes. In those studies manipulation of the surface charge density was performed using ionized surfactants and divalent metal cations [1] changing phospholipid composition [2], or by in vivo treatment with cuprizone [3]. However, such experiments only mimick alterations of the surface charge which might occur under physiological conditions. From this point of view phosphorylation of membrane proteins seems to be the best candidate responsible for changing the surface potential in the living cell. In fact, protein kinases and their substrates are widely distributed in biological membranes.

In [4], we demonstrated a close relationship between protein phosphorylation, changes of the surface potential and changes of apparent K_m-values of certain enzymes in microsomal membranes. Here, we describe similar studies on mitochondrial membranes. Monoamine oxidase (EC 1.4.3.4), an enzyme located in the outer mitochondrial membrane [5], was used as a target, since its susceptibility to changes of the surface potential has already been observed [1–3,6].

Abbreviations: ANS, 8-anilino-1-naphthalene sulphonate; CCCP, carbonyl cyanide-*m*-chlorophenylhydrazone; cAMP, cyclic 3′:5′-adenosine mononucleotide

2. MATERIALS AND METHODS

Mitochondria from rat liver were isolated as in [7]. Mitochondria depleted of the outer membrane (mitoplasts) were prepared using digitonin as in [8]. To obtain outer membranes, the post-mitoplast supernatant was centrifuged at 150000 × *g* for 45 min. Outer membranes were also isolated as described in [9]. The cytosolic fraction was obtained by high-speed centrifugation (100000 × *g* for 60 min) of the post-mitochondrial supernatant. Partially purified cytosolic protein kinase was prepared as in [10].

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Phosphorylation of membrane proteins was carried out at 30°C in a medium containing 250 mM sucrose, 10 mM Tris–HCl (pH 7.4), 0.5 mM ethylene glycol-bis-(2-aminoethyl ether)-*N,N'*-tetraacetate (EGTA), 2 mM $MgCl_2$, 1 mM ATP, oligomycin 2 μg/ml, 3 μM carboxyatractyloside and 3 μM CCCP. Mitochondria or mitoplasts were added to make the protein concentration 1–2 mg/ml and the outer membrane concentration 0.1 mg protein/ml. The phosphorylation was stopped by addition of EDTA to the final concentration of 5 mM, and aliquots were immediately taken for measuring the binding of fluorescent probes or the activity of monoamine oxidase. When protein phosphorylation had to be measured, [γ-^{32}P]ATP (200 cpm/pmol) was used and the reaction was terminated with 10% trichloroacetic acid containing 10 mM KH_2PO_4 and 1 mM ATP (unlabelled). The precipitated protein was washed with 5% trichloroacetic acid followed by ethanol and ethyl ether, and then solubilized in 1 M NaOH. Čerenkov radiation was measured in a Packard scintillation spectrometer [11].

The affinity (the dissociation constant, K_d) of the membranes for ANS and ethidium bromide was measured fluorometrically as in [1,4] using the following excitation and emission wavelengths: for ANS 366 nm and 460 nm, and for ethidium bromide 510 nm and 620 nm, respectively. Monoamine oxidase was measured with dopamine as substrate as in [12]. [γ-^{32}P]ATP was synthesized as in [13].

3. RESULTS

Fig.1 shows the time course of protein phosphorylation in intact mitochondria and mitoplasts. As in microsomes [4], the maximum incorporation of ^{32}P was observed after 10 min incubation. The phosphorylation was much enhanced when the cytoplasmic fraction and cAMP were added (fig.1). The cytoplasmic fraction alone had a much smaller effect (not shown).

In all these experiments phosphorylation of matrix proteins was prevented by the presence of carboxyatractyloside, a strong inhibitor of the ATP/ADP carrier [14]. Moreover, ATP hydrolysis and the resynthesis of [^{32}P]ATP inside mitochondria was prevented by the presence of oligomycin and CCCP. As a result, only proteins of the

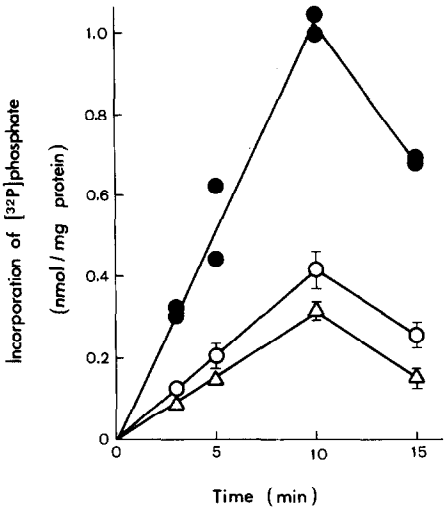

Fig.1. Phosphorylation of mitochondrial membrane proteins. (○) Mitochondria (mean values ± SE from 4 experiments); (△) mitoplasts (mean values ± SE from 4 experiments); (●) mitochondria plus cytosol 0.4 mg protein/ml plus 4 μM cAMP (2 separate experiments). The latter data were corrected for endogenous phosphorylation of cytosolic proteins in the absence of mitochondria, the correction varying between 10% and 20% of decompositions/min measured for the complete sample.

outer membrane, the intermembrane compartment and the inner membrane could be phosphorylated. Subfractionation of the mitochondria after incubation with [^{32}P]ATP revealed that both membranes were labelled to approximately the same extent, about 400 pmol P/mg protein during 10 min, whereas labelling of intermembrane proteins was negligible. Addition of the cytoplasmic fraction, or partially purified cytoplasmic protein kinase, and cAMP increased the incorporation of ^{32}P into proteins of both the outer and the inner membrane, with a preference for the former. Isolated outer membranes became phosphorylated only if incubated in the presence of the cytoplasmic fraction (or partially purified cytoplasmic kinases) and cAMP.

To evaluate changes of the surface potential, dissociation constants for ANS and ethidium bromide were determined along with measurements of membrane protein phosphorylation. It appeared that K_d for ANS increased, whereas K_d for ethidium bromide decreased following the en-

dogenous phosphorylation in both mitochondria and mitoplasts (table 1). However, when either ATP or Mg^{2+} was omitted in the incubation medium, K_d-values remained unchanged (not shown). It has to be stressed that the fluorescence intensity in these experiments was not affected by the energy state of mitochondria, since the media for incubation with ATP and for fluorescence measurements contained CCCP. Thus, changes of K_d for the fluorescent probes could be ascribed solely to changes of the surface potential resulting from protein phosphorylation (cf. [4]). The latter changes ($\Delta\Psi_s$) can be calculated using the formula [1–4]:

$$\Delta\Psi_s = \frac{kT}{z\epsilon} \ln \frac{K_d''}{K_d'} \quad (1)$$

where:

T = the absolute temperature;
k = the Boltzmann constant;
ϵ = the elementary charge;
z = the number of charges on the probe molecule (assumed as -1 for ANS and $+1$ for ethidium bromide);
K_d' and K_d'' = apparent dissociation constants before and after phosphorylation, respectively.

Changes of the surface potential calculated in this way are shown in fig.2. Comparison with fig.1 reveals that the surface potential is changed in parallel with the extent of phosphorylation of membrane proteins.

Concomitantly with the phosphorylation of membrane proteins and the accompanying increase of the negative surface potential, we observed a decrease of the apparent K_m-value of monoamine oxidase, whereas V_{max} of this enzyme remained virtually unchanged (fig.3). These effects were stimulated by cytoplasmic protein kinases and cAMP. Lowering of apparent K_m to half of its initial value, i.e., from 0.34 ± 0.02 mM to 0.19 ± 0.03 mM (mean values ± SE from 5 experiments), occurred after 10 min incubation with ATP and Mg^{2+} alone, whereas the same effect in the presence of the cytoplasmic fraction and cAMP was observed already after 2 min (fig.3).

Assuming that these alterations of apparent K_m-values result from local changes of the substrate concentration due to electric repulsion or attraction of substrate molecules in the immediate vicinity of the membrane [15], changes of the surface potential can be calculated from the formula [1–4]:

$$\Delta\Psi_s = \frac{kT}{z\epsilon} \ln \frac{K_m''}{K_m'} \quad (2)$$

where:

K_m' and K_m'' = apparent Michaelis constants before

Table 1

Effect of endogenous protein phosphorylation on the binding of ANS and ethidium bromide to mitochondria and mitoplasts

Particles	Incubation time in the phosphorylation medium (min)	K_d for ANS (μM)	K_d for ethidium bromide (μM)
Mitochondria	0	18.5 ± 0.4	8.1
	5	22.5 ± 0.5	6.2
	10	27.5 ± 0.9	5.3
	15	26.0 ± 0.6	5.7
Mitoplasts	0	16.2 ± 1.1	
	5	20.2 ± 1.3	
	10	26.2 ± 1.6	

For ANS mean values ± SE from 4 experiments, and for ethidium bromide mean values from 3 experiments are shown

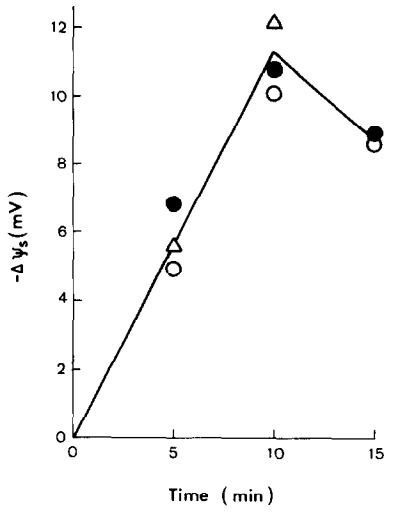

Fig.2. Effect of protein phosphorylation on the surface potential. Changes of the surface potential ($\Delta\Psi_s$) were calculated from mean values of changes of K_d for ANS (○ and △) and ethidium bromide (●) shown in table 1; (○, ●) mitochondria; (△) mitoplasts.

and after phosphorylation of membrane proteins, respectively.

The charge of the substrate molecule dopamine (z) is assumed to be +1. Substituting mean values

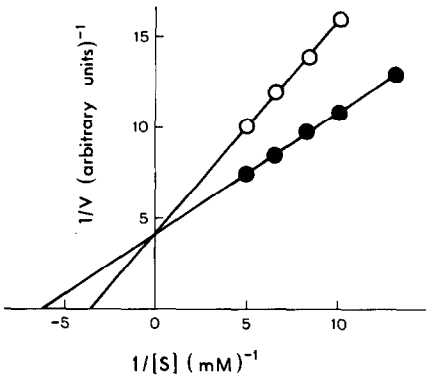

Fig.3. Effect of phosphorylation of membrane proteins on kinetic parameters of monoamine oxidase: Lineweaver-Burk plot. Mitochondria were incubated for 2 min in the medium described in section 2 with the cytoplasmic fraction (0.4 mg protein/ml) and 4 μM cAMP (●). A control sample was incubated in the absence of ATP (○). Apparent K_m-values were 0.277 mM for the control and 0.156 mM for phosphorylated membranes, which correspond to $\Delta\Psi_s$ of −14.7 mV upon phosphorylation.

of apparent K_m before and after incubation of mitochondria with ATP one obtains −15 mV for $\Delta\Psi_s$ upon membrane phosphorylation (see also legend to fig.3 for an individual experiment).

Solubilization of mitochondrial membranes with non-ionic detergents (e.g., Lubrol WX) increased the apparent K_m of monoamine oxidase (see also [6]) and the difference in apparent K_m between phosphorylated and non-phosphorylated membranes completely disappeared.

4. DISCUSSION

Endogenous phosphorylation of mitochondrial proteins has already been described [16–18]. Subfractionation of liver mitochondria has shown that both the outer and the inner membranes are phosphorylated [19], whereas protein kinases are present in the intermembrane compartment [19] and the inner membrane [19,20]. In isolated mitochondria outer membrane proteins are presumably phosphorylated by the intermembrane protein kinase [19], while in the cell, cytoplasmic kinases also contribute to this process. Since both cytoplasmic and intermembrane protein kinases are activated by cAMP [19], it can be presumed that the phosphorylation of mitochondrial outer membrane proteins can be controlled by this nucleotide. In fact, injection of glucagon or butyryl cAMP greatly stimulates in vivo phosphorylation of liver mitochondrial proteins in rat [21]. Consequently, it may be expected that mitochondrial monoamine oxidase activity can be influenced by hormones acting through cAMP.

Although the change of the surface potential upon phosphorylation, calculated from the change of apparent K_m-value of monoamine oxidase, is somewhat higher than calculated from K_d for fluorescent probes, it should be emphasized that monoamine oxidase senses the surface potential of the outer membrane only, whereas fluorescence measurements randomize changes of the surface potential of both mitochondrial membranes.

The effect of protein phosphorylation on monoamine oxidase is, most likely, an indirect one and due to changes of the overall surface potential of the outer membrane, not to phosphorylation of the enzyme molecule itself. This is indicated by the fact that this effect disappears when the phosphorylated membrane is solubilized.

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