EFFECT OF THE PHOSPHORYLATION OF MICROSOMAL PROTEINS ON THE SURFACE POTENTIAL AND ENZYME ACTIVITIES

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1. Introduction

It has been demonstrated [1] that altering the surface charge density of biological membranes changes the activity of membrane-bound enzymes at suboptimal substrate concentrations. This was shown by an increase or a decrease of the app. K_m values, the activity at infinite substrate concentration remaining unchanged. It has been postulated that a change of the app. K_m reflects an alteration of the charged substrate concentration in the immediate vicinity of the membrane, due to electric attraction or repulsion. Manipulation of the surface charge density was performed using divalent cations, natural surfactants, as oleate and palmitoyl-CoA, and commercial detergents.

Since intracellular concentrations of fatty acids, acyl-CoA and divalent cations vary under specific conditions, it may be speculated that the surface charge plays a certain regulatory role. Looking for other physiological mechanisms of changing the surface charge density of cellular membranes, we focussed our attention on phosphorylation of membrane proteins by protein kinases. It has been shown [2–9] that several membraneous structures, like the endoplasmic and the sarcoplasmic reticulum, mitochondria, synaptosomes and the plasma membrane, can be phosphorylated by solubie and membrane-bound (endogenous) protein kinases. The present investigation shows that such phosphorylation alters the activity of certain membrane enzymes in a similar way as a change of the surface charge by means of surfactants or divalent cations.

Abbreviation: ANS, 8-anilino-1-naphthalene sulphonate

2. Materials and methods

2.1. *Microsomes*

Livers of albino rats were homogenized in the medium containing 225 mM mannitol, 75 mM sucrose, 1 mM EDTA, 0.5 mM dithiothreitol and 3 mM Tris–HCl (pH 7.5) and the postmitochondrial supernatant (16 000 × *g* for 20 min) was centrifuged at 90 000 × *g* for 90 min. The resulting microsomal pellet was suspended in 250 mM sucrose.

2.2. *Phosphorylation of microsomal proteins*

Microsomes, ~1 mg protein/ml, were incubated at 37°C in the medium containing 250 mM Tris–HCl (pH 7.5), 2 mM $MgCl_2$ and 1 mM ATP (unless indicated otherwise) containing [γ-^{32}P]ATP, 5 × 10^5 cpm/ml. Aliquots of 0.1 ml were inactivated by 10% trichloroacetic acid and filtered through Millipore filters, 0.8 μm pore size. The filters were washed subsequently with 60 ml cold 10% trichloroacetic acid, followed by 10 ml ethanol and 10 ml ethyl ether. Dried filters were placed in water-filled scintillation vials and the radioactivity was counted by Čerenkov radiation [10].

2.3. *Binding of ANS*

Microsomes were suspended in the same medium as for the phosphorylation, except that unlabelled ATP was used and the medium was supplemented with 5 mM EDTA, and placed in a fluorimeter cuvette thermostated at 37°C. ANS (10 mM) was added in 5 μl portions and the resulting fluorescence was recorded using Perkin-Elmer model MPF-3L spectrofluorimeter operated at 366 nm excitation and 460 nm emission wavelengths. The association constants of

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ANS to the membranes were calculated from the double reciprocal plots of fluorescence intensity versus ANS concentration [1].

2.4. *Enzyme activities*

Arylsulphatase C (EC 3.1.6.1) was determined as in [11]. After preincubation of microsomes in the medium for membrane phosphorylation, EDTA was added to 5 mM final conc., followed by *p*-nitrophenyl sulphate. Incubation was for 5 min at 37°C. Dimethylaniline oxidase (EC 1.14.13.8) was measured according to [12], except that 1 mM NADPH was used instead of the NADPH-regenerating system. Incubation was for 5 min at 37°C.

2.5. *[γ-^{32}P]ATP*

This was synthesized as in [13].

3. Results and discussion

3.1. *Phosphorylation of microsomal proteins*

As shown in fig.1, [^{32}P]phosphate was incorporated into microsomal proteins upon incubation of microsomes with [γ-^{32}P]ATP in the presence of Mg^{2+}. In the presence of 1 mM ATP, the labeling attained its maximum after 10 min, then declined. This decline was probably due to prevailing activity of protein phosphatases under conditions when a great part of ATP was exhausted by a concomitant ATPase and therefore the phosphorylation of membrane proteins

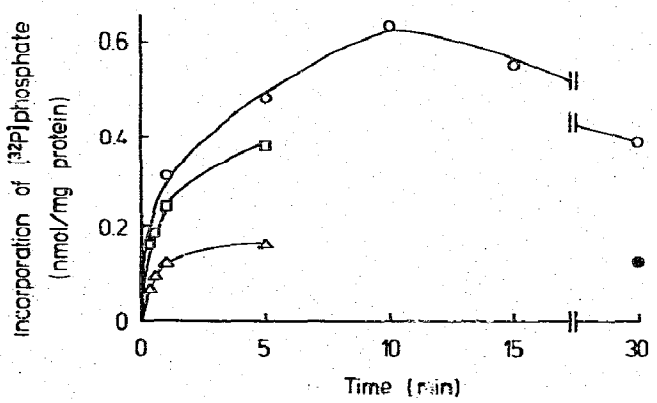

Fig.1. Phosphorylation of microsomal proteins. Initial ATP was: 0.2 mM (△); 0.4 mM (□); 1.0 mM (○, ●). The closed circle indicates a sample to which EDTA was added 5 mM after 10 min, to stop further phosphorylation.

decreased. In fact, it was found that ~50% of added ATP was split within the first 10 min of incubation.

Negligible phosphorylation was observed in the absence of Mg^{2+}. When an excess of EDTA was added after 10 min to block further phosphorylation, the decrease of labeling was much faster than in the presence of Mg^{2+}.

3.2. *Effect of phosphorylation of membrane proteins on the membrane surface potential*

Untreated microsomes and microsomes incubated with 1 mM ATP and 2 mM $MgCl_2$ were rapidly titrated with ANS as in section 2. A double reciprocal plot of fluorescence intensity versus ANS allowed to calculate the association constants (K_a) of ANS with the membranes, assuming that the fluorescence of free ANS was negligible as compared to that of membrane-bound ANS. As can be seen (table 1), the K_a value was considerably increased after incubation of microsomes with ATP and $MgCl_2$. Incubation with ATP or $MgCl_2$ alone had essentially no effect.

Applying the Maxwell-Boltzmann distribution to the binding of charged fluorescent probes [14], it is possible to calculate a change of the surface potential ($\Delta\psi$) from the change of the K_a according to the following equation (see also [1]):

$$\Delta\psi = \frac{kT}{ze} \ln \frac{K_a}{K'_a} \qquad (1)$$

where k is the Boltzmann constant, T the absolute temperature, e the electron charge, and z indicates the number of charges on the probe molecule (for

Table 1
Association constants of ANS with microsomes

Additions	K_a (μM) 0 min	5 min	10 min	15 min	30 min
$MgCl_2$ 2 mM	23	24	24	26	27
ATP 1 mM	24	26	26	24	24
ATP + $MgCl_2$	26	33	38	34	29 / 26[a]

[a] EDTA was added to 5 mM final conc. after 10 min to stop further phosphorylation and to promote the dephosphorylation

The data are mean values of 3–6 expt

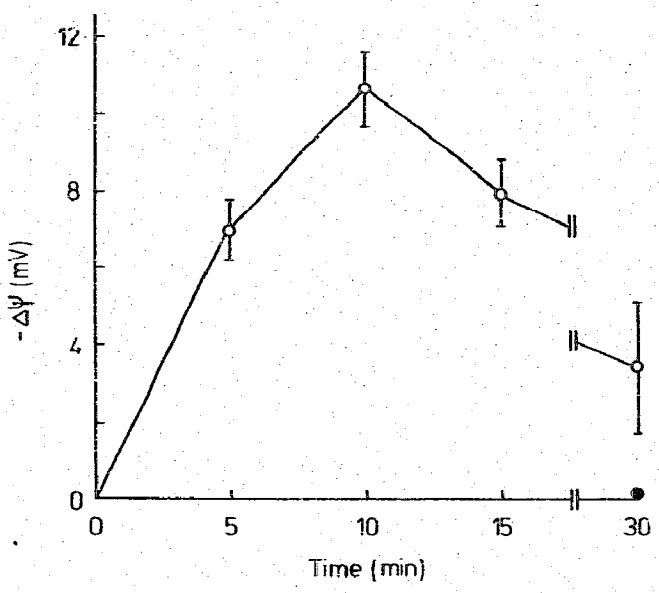

Fig.2. Changes of membrane surface potential upon phosphorylation of microsomes. The values are calculated from K_a values of table 1 using eq. (1). ATP was 1.0 mM. The closed circle corresponds to a sample to which EDTA was added to 5 mM after 10 min to stop further phosphorylation.

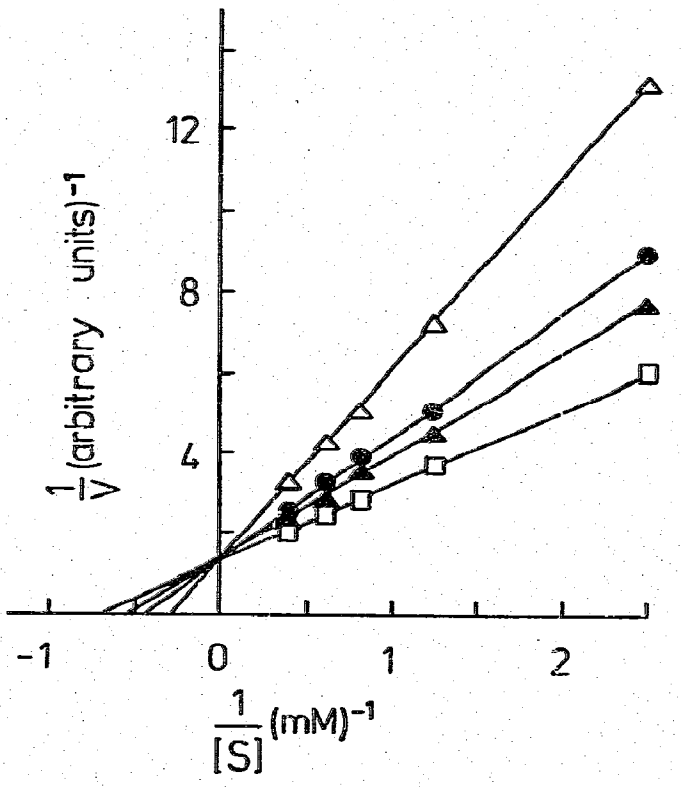

Fig.3. Effect of phosphorylation of microsomal membranes on arylsulphatase activity. (●) Control, incubated in the presence of 2 mM $MgCl_2$ but absence of ATP (K_m 2.3 mM); (△) phosphorylated microsomes, incubated for 10 min with 2 mM $MgCl_2$ and 1 mM ATP (K_m 3.6 mM); (▲) dephosphorylated microsomes, incubated for 10 min with $MgCl_2$ and ATP, followed by 20 min incubation in the presence of 5 mM EDTA (K_m 1.9 mM); (□) microsomes solubilized in Lubrol WX, measured before and after phosphorylation (identical values, K_m 1.5 mM).

ANS, $z = -1$). Thus, performing the titration with ANS at various times of the incubation, it was possible to follow the changes of the surface potential of microsomes during the entire period of phosphorylation and dephosphorylation of membrane proteins. The results (fig.2) show that changes of the surface potential reflect the degree of phosphorylation of membrane proteins (for comparison see fig.1).

3.3. *Effect of membrane phosphorylation on enzyme activities*

After preincubation of microsomes for 10 min under conditions of membrane phosphorylation, i.e., with 1 mM ATP and 2 mM $MgCl_2$, EDTA was added to 5 mM and the activity of arylsulphatase measured immediately. Control samples were preincubated without ATP or $MgCl_2$. Enzyme activity was also determined in samples incubated for 20 min after addition of EDTA, i.e., under conditions when an almost complete dephosphorylation of membrane proteins was achieved (see fig.2). Figure 3 shows that phosphorylation increased the app. K_m value of the enzyme but had no effect on the reaction rate at infinite substrate concentration. Dephosphorylation completely reversed this effect of the phosphorylation. In fact, the app. K_m after dephosphorylation was usually somewhat lower than that of the control. Solubilization of microsomes in Lubrol WX resulted in a substantial decrease of app. K_m arylsulphatase. Solubilized proteins could be phosphorylated to the same extent as those in untreated microsomes if incubated with ATP and $MgCl_2$. However, no change of app. K_m was observed under these conditions. This experiment shows that the effect of protein phosphorylation on arylsulphatase activity is manifested only in intact membranes and disappears when the membrane is solubilized.

In contrast to arylsulphatase, the app. K_m of

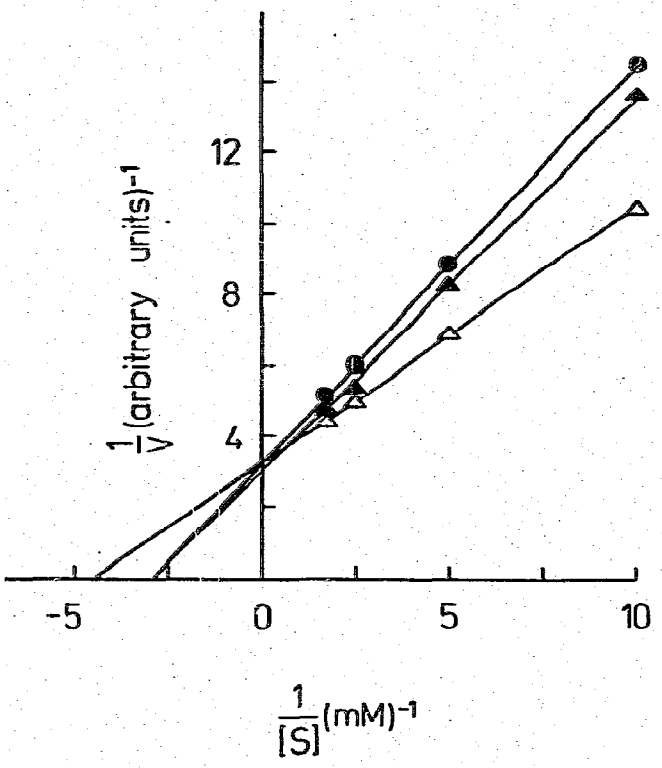

Fig.4. Effect of phosphorylation of microsomes on dimethylaniline oxidase activity. Conditions for phosphorylation and dephosphorylation were as in fig.3. Because Mg^{2+} is required for the enzyme assay, excess of $MgCl_2$ was added to the dephosphorylation sample after 20 min, followed by glucose and hexokinase to trap remaining ATP. (●) Control (K_m 350 μM); (△) phosphorylated microsomes (K_m 244 μM); (▲) dephosphorylated microsomes (K_m 350 μM).

dimethylaniline oxidase was decreased upon phosphorylation (fig.4). In studies not illustrated here, it was observed that app. K_m values of monoamine oxidase (EC 1.4.3.4) of the outer mitochondrial membrane was decreased, whereas that of glycerol-3-phosphate dehydrogenase (EC 1.1.99.5) of the inner mitochondrial membrane was increased under conditions of membrane phosphorylation. It can thus be concluded that membrane phosphorylation, by imposing an additional negative surface charge on biological membranes, increases app. K_m values of several membrane-bound enzymes having anionic substrates and decreases app. K_m of enzymes reacting with cationic substrates.

As discussed in [1], an alteration of the surface potential results in a change of the app. K_m according to the equation:

$$\Delta\psi = \frac{kT}{ze} \ln \frac{K_m}{K'_m} \qquad (2)$$

where K_m denotes the apparent Michaelis constant of the enzyme in untreated (control) particles and K'_m is the constant after phosphorylation; other symbols have the same meaning as in eq. (1). From eq. (2) it can be calculated that phosphorylation of microsomal membranes under conditions adopted in the present study made the surface potential of liver microsomes more negative by ~10 mV (table 2). This value agrees well with that obtained from ANS titration (fig.2).

Table 2
Effect of membrane phosphorylation on the app. K_m values of arylsulphatase and dimethylaniline oxidase

Treatment	Arylsulphatase		Dimethylaniline oxidase	
	K_m (mM)	Δψ (mV)	K_m (mM)	Δψ (mV)
None (control)	2.26 ± 0.22		0.31 ± 0.05	
Phosphorylation	3.73 ± 0.41	−10.6	0.21 ± 0.02	−9.9
Dephosphorylation	1.90 ± 0.15	+4.6	0.31 ± 0.06	0

Changes of the surface potential (Δψ) with respect to the control were calculated using eq. (2). Mean values of 4 expt ± SEM are shown for K_m. Phosphorylation was achieved by 10 min incubation with 1 mM ATP and 2 mM $MgCl_2$ under conditions in section 2. Dephosphorylation was obtained by a subsequent incubation in the presence of 5 mM EDTA. The control sample was incubated without ATP

Thus, phosphorylation of membrane proteins may modulate the activities of certain membrane-bound enzyme by changing the surface charge density. A possible importance of this phenomenon for metabolic regulation requires, however, further elucidation.

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