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INTERACTION OF GLUCOSE OXIDASE WITH PHOSPHOLIPID VESICLES

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SUMMARY

The interactions beween glucose oxidase and phospholipid vesicles were investigated. The investigations were carried on molecules adsorbed on the outer surfaces as well as entrapped in the interior of the vesicles.

The adsorption of glucose oxidase on the surfaces of egg lecithin vesicles, containing varying amounts of cholesterol and stearoylamine was measured by determining the free fraction of glucose oxidase detected in the filtrates.

In general an enhancement of enzymic activity was observed upon interaction with the vesicles. The enhancement depends on the lipid composition of the vesicles and the surface concentration of the adsorbed glucose oxidase. It reached a maximal value at a surface concentration of $1.4 \cdot 10^{11}$ molecules/cm^2 ($\approx 7.1 \cdot 10^4$ Å^2/molecule) on pure phosphatidylcholine vesicles and about $6.5 \cdot 10^{10}$ molecules/cm^2 ($\approx 16 \cdot 10^4$ Å^2/molecule) when the vesicles contained cholesterol or cholesterol and stearoylamine. CD measurements indicated that the change in enzymic activity of the adsorbed glucose oxidase was accompanied by conformational modification of the enzyme.

In order to entrap glucose oxidase into the vesicles, the lipid was sonicated in the presence of the enzyme. After removal of the free and adsorbed enzyme the amount of the entrapped enzyme was determined by measuring its activity after disintegration of the vesicles with Triton. The enzymic activity of the entrapped glucose oxidase served as a measure for the permeability of the bilayer membrane of the lipid vesicles to glucose. Addition of insulin to the suspension of vesicles containing the entrapped glucose oxidase increased the permeability of glucose by up to $9 \cdot 10^{-8}$ cm/s. This value is the lowest estimate based on the assumption that one glucose oxidase molecule was entrapped in every vesicle.

INTRODUCTION

Proteins in general, and enzymes in particular, are either water soluble or bound to biological membranes. The membrane-bound enzymes are inactivated when

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removed from their membrane environment and can be reactivated, in some cases, by interaction with phospholipids [1, 2].

However, the enzymes which are water soluble may also exibit activity when adsorbed on the membrane surfaces [3, 4]. It is therefore of interest to investigate the activity of enzymes interacting with vesicles. The interacting enzymes may be adsorbed on or entrapped in the lipid vesicles or incorporated in the bilayer membrane.

The microenvironment is known to affect the activity of enzymes. Its specific properties are established by the local potentials and pH values, and also by more specific dipole-dipole interactions and local dielectric constants. The local potentials usually affect only the conditions (pH, ionic strength) of maximal activity. However, the specific interactions may change the dielectric microenvironment and introduce more lasting changes in the tertiary and even the secondary structure of the enzymes. The conformation of the enzyme has a gross influence on the active site. Changes in circular dichroism have been observed in polypeptides and proteins interacting with vesicles [5, 6].

Glucose oxidase is not a membranal enzyme, however, as a flavoprotein, it belongs to a group of compounds related to the respiratory chain in the "biological membranes". In this investigation we studied the change in the enzymic activity of this enzyme as well as its circular dichroism (CD) when adsorbed on vesicles of different composition.

In an other set of experiments the enzyme was entrapped in the vesicle and its activity studied across the bilayer membrane of the lipid vesicles. The enzymic activity was then related to the permeability of the lipid membrane to the substrate. This permeability was modified by the addition of insulin.

EXPERIMENTAL

Materials

Glucose oxidase, highly purified grade was obtained from P. and L. Biochemicals, Milwaukee, Wisc. U.S.A. Egg lecithin (grade I) was obtained from Lipid Products (Nutfield, England); cholesterol was purchased from B.D.H. Chemicals Poole, England; stearoylamine from K and K Laboratories, Plainview, N.Y. and insulin crystalline (23–35 I.U./mg) from Fluka AG. Buchs, Switzerland, Triton X-100 from Sigma Chemical Co., St. Louis, Mo. All other reagents were of analytical grade.

Methods

Absorption of glucose oxidase on the vesicle surface. The vesicles were prepared from different lipid mixtures. Lipids of different composition, expressed in molar fractions (Table 1), were dissolved in chloroform and methanol. The organic solvents were driven off by N_2 and the dry phospholipids were suspended in 0.001 M phosphate buffer, pH 7.0 (at concentrations 2–4 mg/ml) by shaking for 5 min on a Vortex mixer. Each suspension was sonicated in a Branson sonifier (Model B 12) at 4 °C under N_2, using a 1 cm titanium tip. After 15 min an almost clear solution was obtained. Traces of titanium metal from the tip and other insoluble materials were removed by short centrifugation at 5000 rev./min. Glucose oxidase was dissolved in the same buffer (0.4–1 mg/ml) and mixed with the vesicles in a total volume of 3 ml on a Vortex mixer for 10 min. Each reaction mixture was kept for 3 h at room temperature.

Unbound enzyme was removed by subsequent washings with the phosphate buffer in an Amicon ultrafiltration cell model 12, using Diaflo membrane X-300. The washing was stopped when no enzymic activity, measured in the filtrate, was detected. The enzymic activity of glucose oxidase bound to vesicles was then determined. The amount of adsorbed protein on the vesicles was determined by the difference between the initial amount of enzyme before interaction with the vesicles, and the free protein found in the filtrate by enzymic and analytical methods. The free protein, as determined by two methods to be described below, was in good agreement, and the bound enzyme calculated by difference agreed fairly well with its direct analytical determination. The results of the adsorption experiments are summarized in Table I.

Entrapment of glucose oxidase into vesicles. Lipid suspensions were prepared as described above. Glucose oxidase, 0.2–0.4 mg/ml, was then added and each reaction mixture was sonicated for 5 min.

After sonication, the suspension (which became almost clear) was passed through an Amicon ultrafiltration cell model 12 for removal of the non-entrapped enzyme, using Diaflo membrane X-300. For the elution of the enzyme adsorbed on the outer surface, the vesicles in the filtration cell were washed with 0.1 M phosphate buffer, then with 10^{-4} M polyglutamic acid solution, and finally again with 0.1 M phosphate buffer. The amount of entrapped enzyme was determined after disintegration of the vesicles with Triton X-100. After every step the total amount of not entrapped (free or adsorbed) enzyme was determined by measuring the enzyme activity in the suspension, while the free enzyme was determined in the filtrate.

Determination of free and latent enzymic activity. The enzymic activity of free and adsorbed glucose oxidase was determined by a modified method of Swoboda [7] omitting the use of catalase. The oxidation of glucose was followed by monitoring the initial rate of oxygen uptake, using a Clark O_2 electrode Model YSI (Yellow Spring Instruments). The assay was carried out at pH 6.2. Latent glucose oxidase activity was determined after liberation of the entrapped enzyme by incubation with a 1 % solution of Triton X-100 at room temperature for 30 min.

Determination of protein bound to vesicles. The quantity of the adsorbed or entrapped protein was determined by the Lowry method [8] using bovine serum albumin as standard, and by amino acid analysis according to Spackman et al. [9], using a Beckman automatic amino acid analyser.

Measurement of the enhancement of membrane permeability to glucose by insulin. 2-ml samples of protein (120 μg) entrapped into vesicles ($\approx$ 20 μg glucose oxidase/mg lipid were incubated for different periods of time with different amounts of insulin (20–50 μg/ml). The enzymic activity in the reaction mixture was determined as described. The increased enzymic activity was found to be directly related to the facilitated transport of the glucose by insulin. The minimal permeability coefficient was calculated from the activity enhancement assuming that only one enzyme molecule can be entrapped in a vesicle, as follows:

$$P = \frac{(\mathrm{d}c_g/\mathrm{d}t)}{(c_p c_g \sigma)} \tag{1}$$

where P is the permeability coefficient given in cm/s, c_g is the glucose concentration, c_p is the enzyme concentration, σ is the area per vesicle. Assuming the improbable

that there are no empty vesicles and that their number is equal to the glucose oxidase molecules, their calculated area would be about $3.5 \cdot 10^{-11}$ cm^2 corresponding to the area of a sphere of about 330 Å in diameter. In reality the values of σ should be lower and the corresponding permeability coefficient P higher.

Circular dichroism measurements. The circular dichroism spectra of the adsorbed glucose oxidase were measured 6 h after preparation, with a Cary Model 60 spectropolarimeter equipped with the model 6002 CD attachment. The enzyme concentrations were between 50 and 350 μg/ml and cells of 1–10 mm light path length were used. The data, reported as mean residue ellipticity $[\theta]$ in degree · cm^2 · dmol^{-1}, were calculated by assuming a mean amino acid residue weight of 115.

RESULTS AND DISCUSSION

Adsorption of glucose oxidase onto phospholipid vesicles

The interaction of glucose oxidase with the vesicles was studied as a function of their composition and their surface charge density. Egg lecithin, cholesterol and stearoylamine were the vesicles components. The charge density was controlled by the ratio of stearoylamine to lecithin-cholesterol in the vesicles. The mol percentage of stearoylamine in the liposomes ranged between 0 and 40 % (Table I) which corresponds up to one stearoylamine molecule per 100 Å^2. The complexes were prepared by mixing the vesicles with the enzyme at various weight ratios (enzyme/lipid = 1/100 → 1/10) followed by removal of the free enzyme by ultrafiltration. The

TABLE I

Composition of vesicle in molar fractions			Concentration of unbound enzyme (μg/ml)	Surface concentration of bound enzyme	
$X_{Lecithin}$	$X_{Cholesterol}$	$X_{Stearoylamine}$		μg glucose oxidase / mg lipid	molecules/cm^2*
1			50	61	$0.9 \cdot 10^{11}$
1			40	62.5	$1.0 \cdot 10^{11}$
1			20	57	$0.8 \cdot 10^{11}$
1			33	110	$1.6 \cdot 10^{11}$
1			90	130	$1.86 \cdot 10^{11}$
0.8	0.2		15	62.5	$7.56 \cdot 10^{10}$
0.77	0.23		41	17.8	$4.2 \cdot 10^{10}$
0.62	0.38		20	27	$6.4 \cdot 10^{10}$
0.62	0.38		16	40	$5.6 \cdot 10^{10}$
0.62	0.38		10	104	$1.48 \cdot 10^{11}$
0.82	0.17	0.01	50	120	$1.68 \cdot 10^{11}$
0.81	0.17	0.02	15	72	$1.0 \cdot 10^{11}$
0.80	0.16	0.04	10	115	$1.6 \cdot 10^{11}$
0.81	0.16	0.03	10	130	$1.84 \cdot 10^{11}$
0.80	0.16	0.04	<5	140	$1.96 \cdot 10^{11}$
0.69	0.13	0.18	<5	130	$1.84 \cdot 10^{11}$
0.57	0.12	0.13	<5	120	$1.69 \cdot 10^{11}$

* Assuming that 65 % of the lipids is on the exterior of the vesicle each lipid molecule occupies approx. 50 Å^2 and molecular weight of glucose oxidase is $15 \cdot 10^4$.

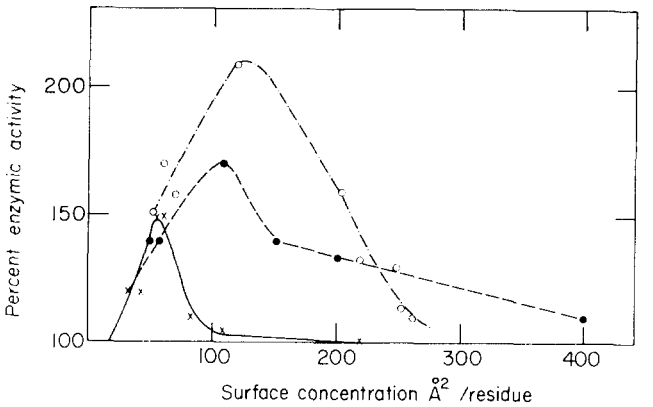

Fig. 1. The relative enzymic activity of adsorbed glucose oxidase on the vesicles of different composition as a function of surface concentration. Vesicle composition: ×–×, lecithin; ●– – –●, lecithin-cholesterol at ratios between 4/1 and 5/1 and ○–.–○, lecithin-cholesterol at the same ratios with 2.5 % stearoylamine added.

adsorption equilibrium relations are presented in Table I. The enzymic activity of adsorbed enzyme on the vesicles of different composition was determined and the results are presented in Fig. 1.

It is evident from Fig. 1 that the enzyme, adsorbed on the lipid vesicles is, as a rule, more active than the free form. The surface concentration of the adsorbed enzymes and their activity depend on the lipid composition.

The protein concentration on vesicles which are constituted only of lecithin seems to be higher than on those containing also cholesterol. Addition of stearoylamine causes further increase in the surface concentration. The activity of the adsorbed enzyme seems to be a complex function of the lipid composition and of the surface concentration. Enhancement of the enzymic activity is highest in the presence of the three lipids (Fig. 1).

It can also be seen from Fig. 1 that the enzyme reaches a maximal activity at a surface concentration, depending on the vesicles composition, namely at about 60 $Å^2$ per amino acid residue on pure lecithin vesicles, and at about 130 $Å^2$ per residue on vesicles containing lecithin+cholesterol and lecithin+cholesterol+stearoylamine. Interrelation between the enzymic activity and the surface concentration of glucose oxidase on the vesicles with and without cholesterol, indicates that it depends on enzyme-enzyme interaction modified by the presence of lipid. Presumably cholesterol increases the rigidity of the bilayer membrane and diminishes the lipid-enzyme interaction, which shifts the maximal enzymic activity towards higher surface concentrations. At excessively high surface concentrations, a partial screening of the active sites seems to occur impeding access of the substrate. It is quite clear that the positive surface charge density on the adsorbing vesicle affects the adsorption forces. We tried to study the effect of these adsorption forces on the enzymic activity at approximately constant surface concentrations of the enzyme. The surface charge density is proprotional to the ratio of stearoylamine to the other lipids in the vesicles. In Fig. 2 we plotted the relative enzymic activity as a function of X_{SA}, the molar fraction of stearoylamine (SA) in the vesicles. At each experimental point, the surface concentra-

tion of glucose oxidase is indicated (experimentally it is exceedingly difficult to obtain the same surface concentration). It is evident from Fig. 2 that the enzymic activity reaches a maximal value between $X_{SA} = 0.02$ and 0.2.

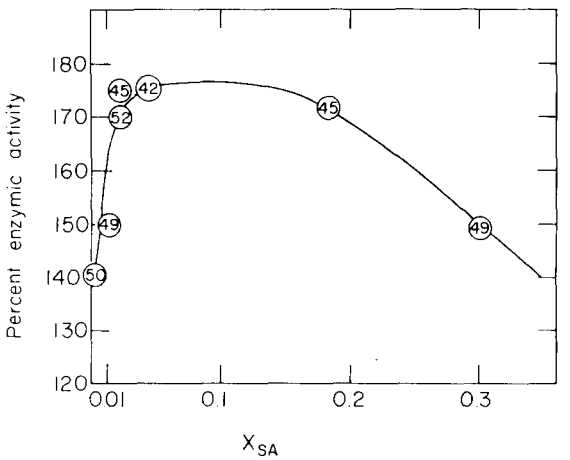

Fig. 2. The relative enymic activity of the adsorbed glucose oxidase as a function of the molar fraction of stearoylamine. The surface concentration is denoted for each experimental point.

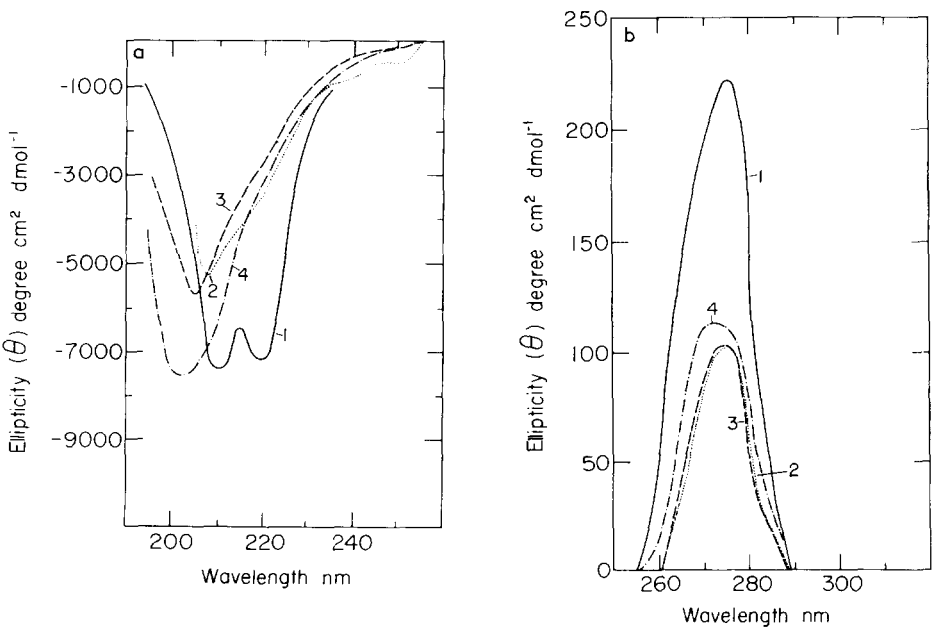

Fig. 3. CD spectra of the free glucose oxidase (—) and adsorbed on the vesicles with different composition; vesicle composition: (. . .), lecithin; – – –, lecithin-cholesterol; –.–.–, lecithin-cholesterol-stearoylamine. (a) CD spectra in the range of 190–240 μm. (b) CD spectra in the range of 250–300 μm.

CD measurements

In Figs. 3a and 3b the molar ellipticity of free glucose oxidase in aqueous solution, and that interacting with vesicles of different composition, is given as function of wavelength.

In Fig. 3a the CD spectra in the far ultraviolet region are presented. The ellipticity in this region is caused by the peptide chain and is sensitive to its secondary structure. Glucose oxidase in the free form shows as CD spectrum characteristic of an α-helix. The molar ellipticity at 220 nm indicates that about 20–25 % of the protein chain is in the α-helical form. The rest of the molecule is partly in the random configuration and partly in the β-structure. Upon interaction with the vesicles, most of the α-helix disappears and the negative ellipticity at 220 nm is strongly reduced. The superposition of the random coil and β-structures cause a shift in the position of the maximal negative ellipiticity toward the lower wavelengths. A similar shift, even with larger residual α-helicity has been observed upon interaction of glucose oxidase with sodium dodecyl sulfate [10].

At 275 nm (Fig. 3b) a weaker positive peak is observed. The size of the peak is further reduced upon interaction of glucose oxidase with the vesicles.

Glucose oxidase entrapment in vesicles

Formation of vesicles in the presence of glucose oxidase results in a partial entrapment of the enzyme. Removal of the adsorbed enzyme from the surface of vesicles was carried out by repeated washings with (0.1 M) phosphate buffer or polyglutamic acid (10^{-4} M in monomeric units of glutamic acid). The enzymic activity was determined after each washing. Residual enzymic activity was detected even after repeated washings of the vesicles. This residual activity may stem from non-displaced enzyme on the surface as well as from the activity of the entrapped enzyme which is controlled by the rate of diffusion of the glucose (substrate) across the bilayer membrane

It can be seen from Table II that the presence of stearoylamine in the composition of vesicles is an essential factor for the entrapment of the enzyme. The entrapment of glucose oxidase in the vesicles occurres only in the presence of stearoylamine and seems to depend also on the ratio of cholesterol to lecithin. This last dependence is subject to further investigation. Most of the enzymic activity of the entrapped glucose oxidase is in the "latent" form and its full activity can be measured only by breaking up the vesicle which was carried out by incubation with Triton X-100. This detergent is non-inhibitory to the enzyme activity at the concentrations used for its determination. Control experiments, carried out by us showed that the enzymic activity of glucose oxidase was not affected even by a 3-fold higher concentration of Triton, than used for vesicle disintegration.

According to Table II, the amount of protein entrapped is dependent on the lipid composition. However, in all the cases when sufficient stearoylamine was present, the amount of glucose oxidase entrapped varied between 12 and 32 μg glucose oxidase per mg of lipid. Taking 22 μg/mg as an average value we can calculate the size of the vesicle, assuming that each vesicle accomodates one protein molecule. The molecular weight of such a vesicle would be about $7.5 \cdot 10^6$ which corresponds to about 340 Å for its outer diameter and about 230 Å for its inner diameter. These values are in fair agreement, although somewhat larger, with those obtained for empty bilayer vesicles

TABLE II

ENTRAPMENT OF GLUCOSE OXIDASE INTO LIPOSOMES

Enzymic activity was expressed in arbitrary units corresponding to instrument readings. For calculation of the three last columns the enhancement estimate introduces an error of about 10 % in the values given.

Composition of liposomes	Molar fractions of the components of liposomes	Enzymic activity after sonication	Enzymic activity of the adsorbed enzyme	Residual enzymic activity after repeated washings	Enzymic activity after incubation with Triton	Protein entrapped		
						μg protein / mg lipid	Percent of total protein entrapped	Protein entrapped / protein adsorbed
Phosphatidylcholine	1	370	300	100	70	—		0
Phosphatidylcholine+cholesterol	0.62+0.38	260	200	60	40	—		0
Phosphatidylcholine + cholesterol +stearoylamine	0.86+0.13+0.002	900	400	100	90	~1	3	~0.05
Phosphatidylcholine+cholesterol +stearoylamine	0.69+0.28+0.03	70	50	40	80	12.7	45±5	1.3 ±0.2
Phosphatidylcholine+cholesterol +stearoylamine	0.57+0.28+0.15	185	160	35	100	23.6	35±5	0.8 ±0.1
Phosphatidylcholine + cholesterol +stearoylamine	0.64+0.27+0.09	560	490	90	350	20	40±5	0.95±0.1
Phosphatidylcholine+cholesterol +stearoylamine	0.51+0.35+0.14	470	350	140	280	19.6	23±3	~1 ±0.1
Phosphatidylcholine+stearoylamine	0.88+0.12	960	870	100	460	32	36±5	~0.65±0.1

through sonication. Vesicles of this size are not likely to accomodate more than one molecule of 150 000 molecular weight. However, under the sonication conditions at inadequate excess of lipids with respect to glucose oxidase, a large part of the vesicles may be left as empty bags. In this case, the number of vesicles formed is larger than the number of entrapped molecules, which would make their calculated size smaller. It is interesting to note in Table II that the ratio of entrapped enzymes to those adsorbed on the outer surface of stearoylamine-containing vesicles is generally larger than 0.5, which is approximately the inside to outside surface area ratio. This seems to indicate that the protein interaction with a lipid mixture containing stearoylamine favors concave-shaped lipid surface formation around the protein molecule. This contradicts the reports on the entrapment of lysozyme [11] of amyloglucosidase and of albumin [12] where it is claimed that identical charge on the protein and on the liposomes favors entrapment, while opposite charge favors adsorption on the outer surface. Indeed, in spite of the adsorption on the outer surface, the fraction of the proteins entrapped in these experiments are considerably higher (up to 45 %) than in the experiments [11, 12] reported previously. Recently, however, every high entrapments of glucose oxidase in negatively charged liposomes under unspecified conditions were reported [13].

The effect of insulin on the permeability of vesicles to glucose

Incubation with insulin of vesicles containing entrapped glucose oxidase results in the increase of the permeability of the vesicles to glucose. This effect is demonstrated by an increase of enzymic activity of the glucose oxidase entrapped in the vesicles. The measurement of the enhancement of the permeability coefficients of glucose was performed by determining enzymic activity of the entrapped enzyme after incubation with various concentrations of insulin. The results are summarized in Fig. 4. The insulin concentration varied between 20 and 50 μg/ml and the enzymic activity was measured after different times of exposure of the vesicles to the insulin.

Control experiments showed that insulin, besides facilitating glucose transport, tends to remove the glucose oxidase from the vesicle surface. Most of the residual glucose oxidase which remained on the vesicles after repeated washings, was displaced through incubation with insulin and was detected in the ultrafiltrate. Since the activity of the adsorbed glucose oxidase is enhanced, its removal from the surface results in a decrease of its contribution to the total enzymic activity. This is demonstrated in Fig. 4b where the fraction of initial enzymic activity of adsorbed glucose oxidase is given as a function of incubation time with insulin. Up to about an hour, the decrease of activity enhancement is practically linear. The initial enhancement is about 70 %, it decreases to about 30 % after 1 h and it is completely abolished after several hours.

It can be seen that the enzymic activity was lowered to values corresponding to the unbound enzyme, indicating that the enzyme is displaced from the lipid environments. Incubation of unbound enzyme with insulin, even at high ratio of insulin to glucose oxidase does not decrease enzymic activity.

The increase in enzymic activity due to facilitated glucose transport by insulin as a function of its concentration and time of incubation is given in Fig. 4a. In one group of curves the net increase in activity seems to result from the facilitated transport. In the other group of curves the decrease of activity enhancement caused by the displacement of the adsorbed glucose oxidase (as indicated in part b of the figure)

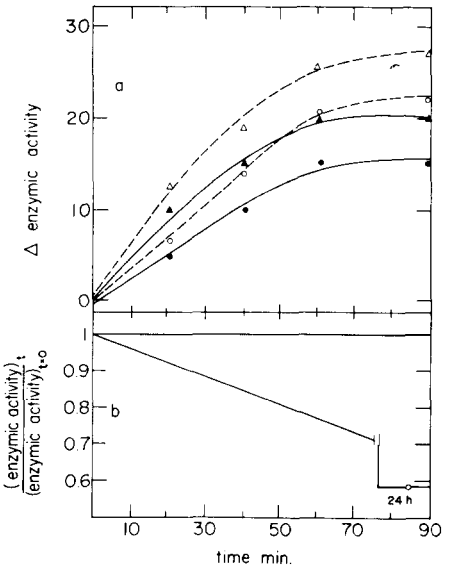

Fig. 4. Effect of insulin on the enzymic activity. (a) The enhancement of enzymic activity of entrapped glucose oxidase in the presence of insulin (circles, 20 μg/ml; triangles, 50 μg/ml) as a function of time. Solid lined (filled symbols) measured increment in enzymic activity. Broken lines (open symbols) calculated increment by facilitated transport, taking into account the enhancement lowering with displacement of the adsorbed enzyme by insulin as shown in b. (b) Lowering of the enhanced enzymic activity of glucose oxidase adsorbed on the vesicles by added insuling (10 μg/ml) as function of time.

is taken into consideration. Depending on insulin concentration, time of exposure and the above mentioned assumptions concerning the displacement of residual adsorbed glucose oxidase the calculated increment in permeability coefficient reached values up to $9 \cdot 10^{-8}$ cm/s (Fig. 4a). The permeability coefficient (P) was evaluated with the aid of Eqn. 1 assuming a perfect match between the number of vesicles and entrapped protein molecules. If, however, only part of the vesicles are filled with protein molecules and the rest be empty bags, the calculated value of P would be larger, and considerably closer to those obtained by Kafka [14] for planar bilayers of lecithin. E.g. taking 210 Å for the outer diameter [15] of the entrapped vesicles, the calculated increment in P would be about $2 \cdot 10^{-7}$ cm/s.

Conclusions

(1) The enzymic activity of glucose oxidase depends on its microenvironment. It changes its microenvironment during adsorption from aqueous solution onto the surfaces of phospholipid vesicles. The new microenvironment affects both the enzymic activity and the conformation, as determined by CD measurements. However, the CD is an indicator mainly of the secondary structure and only indirectly of the tertiary structure of the protein and of the FAD apoprotein bonding. Unlike enzymic activity, CD depends only little on the composition of the adsorbing vesicles. The enzymic activity depends also on the surface concentration, having an optimal activity at a surface concentration corresponding to an area between 60 and 140 Å^2 per amino acid

residue, depending on the lipid composition. This indicates that there is an interrelation between the enzymic activity and the protein-protein and protein-lipid interactions. Thus the microenvironment is a complex function of these interactions involving Coulombic and hydrophobic forces. The protein molecule adsorbed on the vesicles surface may penetrate the lipid layer to different extents. The degree of penetration may be a function of protein-lipid and protein-protein interactions of the surface of the vesicle.

(2) A high degree of entrapment of glucose oxidase in bilayer lipid vesicles (up to 45 % of the glucose oxidase molecules) can be achieved by sonication of glucose oxidase with egg lecithin, cholesterol and stearoylamine. The high degree of entrapment indicates that a large portion, if not the majority of the vesicles contain entrapped enzymes, suggesting that during sonication the vesicles are formed over glucose oxidase molecules as nuclei.

(3) The activity of the entrapped enzyme is controlled by the permeability of the vesicle membrane to the substrate and to the products. It was shown that the permeability coefficient can be determined by measuring the enzymic activity, provided that the size of the vesicles and the number of encapsulated enzymic molecules per vesicle are known.

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