The use of proteinases to determine the topological location of cytochrome *P*-450 in vesicles derived from smooth endoplasmic reticulum of rat liver

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1. The phospholipid bilayer of intact vesicles from smooth endoplasmic reticulum is impermeable to macromolecules. Specific and non-specific proteinases were used to investigate the site of membrane proteins in the transverse plane of the bilayer. 2. When two proteinases were used in conjunction, denaturing effects additional to proteolysis were observed on cytochrome *P*-450 content and glucose 6-phosphatase activity which did not depend on the integrity of the phospholipid bilayer. 3. When lipid peroxidation was inhibited, these effects were not observed.

The distribution of proteins in the transverse plane of the phospholipid bilayer of vesicles derived from microsomal membranes can be investigated by the use of proteinases (Ito & Sato, 1969; De Pierre & Dallner, 1975; Nilsson & Dallner, 1977). These enzymes cannot penetrate the phospholipid bilayer, and thus only those proteins with susceptible bonds exposed on the external face of the vesicles are degraded. As examples of this methodology, it has been established that glucose 6-phosphatase (EC 3.1.3.9) is located on the inner (luminal) face of microsomes (microsomal fraction), whereas cytochrome *b₅* and its associated NADH-dependent reductase (EC 1.6.99.3) are located on the outer (cytosolic) face. Our previous studies employing trypsin (EC 3.4.21.4) have indicated that cytochrome *P*-450 and ferrihaemoprotein *P*-450 reductase (EC 1.6.2.4) are present on both luminal and cytosolic faces of the vesicles derived from smooth endoplasmic reticulum. Evidence was also obtained that the cytochrome *P*-450 variant induced by phenobarbital is cytosolically orientated (Cooper *et al.*, 1980). The effect of trypsin on cytochrome *P*-450 is to convert it into the catalytically inactive but spectrally detectable form known as cytochrome *P*-420.

The use of proteinases to derive the location of proteins has limitations, since the methodology requires that the protein under investigation possesses bonds that are susceptible to the action of the specific proteinase employed. The accessibility of the proteinase to the sensitive bonds must depend solely on the integrity of the phospholipid bilayer and not on other steric and conformational factors. However, hydrolysis of these bonds must interfere with the functional characteristics of the proteins that are

being assayed. To overcome this problem, a non-specific proteinase, used either alone or in conjunction with trypsin, has been employed to investigate the transverse distribution of cytochrome *P*-450 in microsomes (Nilsson *et al.*, 1978). These workers found that with either proteinase used alone, only a portion of the cytochrome *P*-450 was degraded, whereas when they were used together, most of the cytochrome *P*-450 was lost. On the basis of this synergistic effect, it was concluded that cytochrome *P*-450 is located solely on the cytosolic face of the microsomes. Steric hindrance or the absence of susceptible bonds, or both, was presumed to account for the fact that only partial loss of cytochrome *P*-450 content occurred when proteinases were used individually.

In the present paper we give evidence that effects additional to proteolysis occur when mixtures of proteinases are used to investigate the topology of membrane proteins. As a consequence, membrane proteins may be inactivated by mechanisms not involving proteolytic cleavage, which do not always fulfil the necessary criteria for topological investigations.

Materials and methods

Vesicles from the smooth endoplasmic reticulum of rat liver were prepared from male Sprague-Dawley rats, permitted food and water *ad libitum*, as previously described (Blyth *et al.*, 1971), except that the final membrane pellet was resuspended in a solution containing: KCl (50 mM), MgCl₂ (5 mM) and Tris (50 mM) adjusted to pH 7.5 at 25°C with HCl, by the use of a small glass Dounce homogenizer.

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Phospholipid was extracted from membrane vesicles by the method of Folch *et al.* (1957), and its phosphorus content determined as described by Chen *et al.* (1956).

Lipid peroxidation was monitored by malonaldehyde formation as measured by reaction with thiobarbituric acid (Sinnhuber & Yu, 1958; Stocks & Dormandy, 1971). A sample (200 μ l) containing approx. 800 μ g of microsomal protein was added to a solution containing sodium arsenite (0.4 ml of 0.1 M) in trichloroacetic acid (28%, w/v). Thiobarbituric acid (0.2 ml of 1%, w/v) in NaOH (0.05 M) was added and the volume made up to 1.4 ml with water. After incubation for 10 min at 100°C with occasional mixing, precipitated protein was removed by centrifugation at 10 000 g for 2 min and the A_{535} of the supernatant was measured against a blank similarly prepared but containing a portion of buffer in place of microsomes. The concentration of malonaldehyde present was calculated by assuming $A_{535}^{1\%}$ at 535 nm to be 1.56×10^5 litre $\cdot$ g $^{-1}$ $\cdot$ cm $^{-1}$ (Sinnhuber & Yu, 1958).

Glucose 6-phosphatase activities were determined by the method of Leskes *et al.* (1971), except that incubations were carried out in the membrane-resuspension buffer described above, and that the membrane suspension was not sonicated before the determination; where stated, deoxycholate was included to a final concentration of 0.05%. Cytochrome *P*-450 was determined by the method of Omura & Sato (1964). Since lipid peroxidation causes significant production of CO (Nishibayashi *et al.*, 1967), it was necessary to perform this assay in a modified manner. The contents of both cuvettes were saturated with CO and a baseline spectrum was obtained from 400 to 500 nm. A few crystals of sodium dithionite were added to one cuvette and the spectrum was re-scanned to obtain the difference spectrum. The concentration of cytochrome *P*-450 was calculated by assuming the difference $\epsilon_{450} - \epsilon_{490}$ to be 91 mm $^{-1}$ $\cdot$ cm $^{-1}$ (Omura & Sato, 1964).

Proteolytic digestion of vesicles from the smooth endoplasmic reticulum was carried out by incubation at 25°C for 30 min at a membrane-protein concentration of 4 mg/ml in the same medium in which the membrane was previously resuspended. Sucrose was not present in the incubation medium, since it interferes with the colour reaction for malonaldehyde. Proteinases were included in the incubations at 50 μ g of each enzyme per mg of microsomal protein. After incubation, samples were withdrawn and glucose 6-phosphatase activity, cytochrome *P*-450 content and the extent of lipid peroxidation determined.

Pronase (B grade) (EC 3.4.24.4) was obtained from Calbiochem-Behring Corp., La Jolla, CA,

U.S.A. Trypsin (Sigma type I), chymotrypsin (Sigma type VII; EC 3.4.21.1), non-specific proteinase (Nagarse) from *Bacillus amyloliquefaciens* (Sigma protease type VII) (EC 3.4.21.14), and soya-bean trypsin inhibitor were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K. All chemicals used were of A.R. grade or equivalent. All solutions were prepared with water twice distilled in an all-glass apparatus.

Results

The effects of proteinases on glucose 6-phosphatase and cytochrome *P*-450 in vesicles from smooth endoplasmic reticulum are shown in Table 1(a), together with data indicating the extent of lipid peroxidation during the incubations as measured by malonaldehyde formation. The individual enzymes each degrade approx. 50% of the cytochrome *P*-450. In contrast, under the same incubation conditions, incubation with a combination of trypsin plus another proteinase causes up to 75% of the content of cytochrome *P*-450 to be lost.

The specific proteinases trypsin and chymotrypsin cause only a small loss of glucose 6-phosphatase. The non-specific proteinases cause a greater loss of this enzyme, and combinations of proteinases in general cause a greater loss than the sum produced by the separate components.

Table 1(a) shows that all the proteinases used individually cause some lipid peroxidation, although this is relatively small for the specific enzymes. The extent of lipid peroxidation is greater when the non-specific proteinases are used. Table 1(b) shows that the extent of lipid peroxidation is greatly decreased by inclusion of butylated hydroxytoluene in the incubations. The loss of glucose 6-phosphatase by a mixture of trypsin and Nagarse is much decreased, and is thus probably due to lipid peroxidation. In the presence of butylated hydroxytoluene, a mixture of trypsin and Nagarse does not cause any greater loss of cytochrome *P*-450 than does either enzyme used individually. It is reasonable to deduce that the 'extra' cytochrome *P*-450 degraded by this proteinase mixture in the absence of butylated hydroxytoluene is luminally orientated protein which is destroyed by a mechanism involving lipid peroxidation. The fact that cytochrome *P*-450 can be destroyed by lipid peroxidation has previously been reported (Levin *et al.*, 1973). The extent of lipid peroxidation produced by a mixture of trypsin plus another proteinase is approximately the sum of that produced by the constituents. Inhibition of the trypsin component of the mixture by soya-bean trypsin inhibitor added at the start of the incubation lessens the loss of cytochrome *P*-450 and glucose 6-phosphatase and the extent of lipid peroxidation to that observed for the other pro-

Table 1. *Effects of proteinases, Fe²⁺ and butylated hydroxytoluene on cytochrome P-450, glucose 6-phosphatase and malonaldehyde formation in vesicles from the smooth endoplasmic reticulum*

Membranes were incubated with proteinases as described in the Materials and methods section. Fe²⁺, where present, was added as FeSO₄. The results show the means $\pm$ s.d. from four separate experiments. Values for malonaldehyde are the amounts produced during the incubation, which was for 30 min. The quantity of malonaldehyde initially present before incubation is not included. Cytochrome P-450 is expressed as a percentage of that present before incubation; 100% represents a concentration of 78 ± 6 pmol of cytochrome P-450/ μ g of lipid P. Glucose 6-phosphatase activities are similarly expressed; 100% represents an activity of 86 ± 10 nmol of P_i released/min per μ g of lipid P. Abbreviation: BHT, butylated hydroxytoluene.

Addition	Cytochrome P-450 remaining (%)	Glucose 6-phosphatase activity remaining (%)	Malonaldehyde produced (pmol/ μ g of lipid P)
(a) None	100	100	0.0 $\pm$ 0.8
Trypsin	46.7 $\pm$ 3.9	92.7 $\pm$ 4.1	1.98 $\pm$ 0.2
Nagarse	44.0 $\pm$ 2.1	84.5 $\pm$ 4.2	3.7 $\pm$ 0.3
Trypsin + Nagarse	25.0 $\pm$ 5.8	70.1 $\pm$ 3.6	5.5 $\pm$ 0.5
Trypsin + Nagarse + soya-bean trypsin inhibitor	45.0 $\pm$ 3.4	84.5 $\pm$ 4.9	3.5 $\pm$ 0.8
Chymotrypsin	57.1 $\pm$ 3.2	96.1 $\pm$ 2.4	2.24 $\pm$ 0.8
Trypsin + chymotrypsin	38.5 $\pm$ 4.8	74.1 $\pm$ 6.2	4.35 $\pm$ 0.7
Pronase	38.0 $\pm$ 3.8	76.1 $\pm$ 4.8	3.38 $\pm$ 0.34
Pronase + trypsin	25.0 $\pm$ 4.2	41.0 $\pm$ 2.3	4.79 $\pm$ 0.36
(b) BHT (0.02%)	100 $\pm$ 0.9	100 $\pm$ 1.8	0.5 $\pm$ 0.4
Trypsin + BHT (0.02%)	45.3 $\pm$ 3.2	96.3 $\pm$ 3.2	1.1 $\pm$ 0.4
Nagarse + BHT (0.02%)	47.0 $\pm$ 3.1	88.0 $\pm$ 4.1	1.59 $\pm$ 0.4
Trypsin + Nagarse + BHT (0.02%)	45.3 $\pm$ 3.2	85.0 $\pm$ 4.8	1.37 $\pm$ 0.38
Fe ²⁺ (20 μ M)	82.5 $\pm$ 3.2	84.8 $\pm$ 6.4	13.7 $\pm$ 1.4
Trypsin + Nagarse + Fe ²⁺ (20 μ M)	12.2 $\pm$ 3.4	20.1 $\pm$ 4.9	16.2 $\pm$ 2.0
Fe ²⁺ (20 μ M) + BHT (0.02%)	95.0 $\pm$ 4.3	94.7 $\pm$ 2.3	1.20 $\pm$ 0.32

teinase when used individually. It seems therefore that the apparent synergism observed is due to the combined action of the two proteinases and not to a non-proteolytic contaminant present in either preparation. It is also clear from the effects on glucose 6-phosphatase that combinations of proteinases can cause damage to lumenally orientated proteins. This is particularly true for a mixture of Pronase and trypsin. In view of the high susceptibility of glucose 6-phosphatase to trypsin when the accessibility barriers are removed (Cooper *et al.*, 1980), this effect cannot be due to a population of inverted vesicles in the preparation.

Fe²⁺ is known to be a potent initiator of microsomal lipid peroxidation (Poyer & McCay, 1971; Wills, 1966). The concentration of Fe²⁺ could increase owing to release from bound forms during incubation with some of the proteinases. The lipid peroxidation thus initiated could damage proteins present on the luminal side of, or embedded in, the phospholipid bilayer by mechanism(s) not involving proteolytic action by the proteinases.

As shown in Table 1(b), when vesicles from smooth endoplasmic reticulum are incubated with Fe²⁺ very extensive lipid peroxidation is observed and the amounts of cytochrome P-450 and glucose 6-phosphatase are significantly decreased. These decreases are arrested by the inclusion of butylated hydroxytoluene in the incubation.

The possibility was considered that the apparent loss of glucose 6-phosphatase observed on incubation of microsomes with proteinases was due to these enzymes causing a decrease in the permeability of the phospholipid bilayer to the substrate. To eliminate this possibility, glucose 6-phosphatase activities were measured in the presence of 0.05% (w/v) sodium deoxycholate, which renders the phospholipid bilayer permeable to macromolecules (Kreibich *et al.*, 1973) and could make the membranes freely permeable to glucose 6-phosphate. The activity was enhanced in both control and proteinase-treated membranes to the same extent (approx. 55%) by the detergent. There was no differential enhancement of the activity in proteinase-treated membranes. These observations confirm a report in the literature that the activity of glucose 6-phosphatase in intact microsomes is stimulated by the presence of deoxycholate (Nordlie & Arion, 1965). It would appear that the observed action of proteinases on the glucose 6-phosphatase activity of smooth-membrane vesicles is not due to changes in the permeability of the phospholipid bilayer to glucose 6-phosphate, and that the changes in the activity of this enzyme are probably due to the effects of lipid peroxidation rather than proteolysis.

Thus, in spite of the great stimulation of lipid peroxidation by Fe²⁺ in the absence of proteinases, the loss of glucose 6-phosphatase activity is not as

great as when two proteinases are used in conjunction in the absence of Fe^{2+} . This suggests that peroxidation of membrane phospholipids exerts a direct denaturing action and also has some effect on the phospholipid bilayer rendering a population of the vesicles permeable to proteinases.

Discussion

The results presented here demonstrate that effects additional to proteolysis, such as lipid peroxidation, can occur when proteinases are used in combination to establish the location of proteins in the phospholipid bilayer. These effects are sufficient to inactivate partially or denature proteins which are otherwise protected by the phospholipid bilayer from direct proteolysis.

It has been suggested, from results from experiments using combinations of proteinases (Nilsson *et al.*, 1978), that cytochrome *P*-450 is located solely on the cytosolic face of microsomal vesicles. The results presented here are at variance with this interpretation. It is demonstrated that luminally facing proteins can be destroyed by the combinations of proteinases used, by mechanisms other than proteolysis, which probably involve lipid peroxidation. Indeed, it is known that lipid peroxidation causes the loss of haem from cytochrome *P*-450, rendering it spectrally undetectable (De Matteis *et al.*, 1977). Another microsomal haemoprotein, cytochrome *b*₅, can also be partially inactivated by lipid peroxidation (Tappel & Zalkin, 1960). Lipid peroxidation can also cause destruction of the haem of cytochrome *P*-450, with release of Fe^{2+} ions (Levin *et al.*, 1973).

The mechanism by which combined proteinases cause increased lipid peroxidation might involve a primary production of superoxide ions (Fee & Teitelbaum, 1972). However, we were unable to detect the presence of such species as judged by the oxidation of adrenaline. Neither did the addition of superoxide dismutase cause any inhibition of lipid peroxidation (results not shown).

An alternative approach to the establishment of the topology of membrane proteins has been the use of the non-penetrating denaturing agent, *p*-diazobenzenesulphonate. This chemical has a molecular weight of 217 and is thus unlikely to be sterically hindered when reacting with cytosolically orientated proteins (Nilsson *et al.*, 1978). Denaturation is thought to be effected by the reaction of the diazo moiety with thiol, amino and hydroxy groups present in proteins, but it can also react with phospholipid (Nilsson *et al.*, 1978). When used to establish the topology of glucose 6-phosphatase and cytochrome *P*-450, approx. 20% of the former activity and almost all of the cytochrome *P*-450 content is lost. The loss of glucose 6-phosphatase

activity strongly suggests that either the permeability barrier of the phospholipid bilayer has been decreased or that the reactions of the diazo moiety have given rise to lipid peroxidation with its consequent effects on the enzyme content of the microsomes.

The data presented here are consistent with our previous findings that cytochrome *P*-450 has at least three membrane loci (Cooper *et al.*, 1980). In uninduced rats approx. 50% of the cytochrome is cytosolically orientated, with the remainder either luminally facing or embedded in the phospholipid bilayer (Stier *et al.*, 1978). It is known that cytochrome *P*-450 exists in multiple forms with different molecular weights and substrate specificities (Haugen *et al.*, 1975; Bergman & Dallner, 1976; Coon *et al.*, 1976; Haugen & Coon, 1976). Each species could occupy only one of the three loci or, alternatively, any one species of the cytochrome could be distributed between the three sites. The former possibility seems true for the species of cytochrome *P*-450 induced by phenobarbital, since this appears to be predominantly located on the cytosolic face of vesicles from the smooth endoplasmic reticulum (Cooper *et al.*, 1980).

We have shown that glucose 6-phosphatase is also partially, but not completely, inactivated by lipid peroxidation. This could be due to a direct denaturing action of the products of lipid peroxidation on the enzyme and an effect on the phospholipid bilayer rendering a population of the vesicles permeable to proteinases.

The results in this paper clearly demonstrate that great care must be taken in the use of proteinases to determine the topology of membrane proteins if artifactual results are to be avoided.

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