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Cholesterol oxidase as a structural probe of biological membranes: its application to brush-border membrane

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Cholesterol present in intact brush-border membrane vesicles made from rabbit small intestine is a poor substrate for cholesterol oxidase (EC 1.1.3.6, from *Nocardia* sp. and *Nocardia erythropolis*). It becomes susceptible to oxidation by the enzyme only after the addition of detergent, e.g., Triton X-100, in quantities sufficient to disrupt the membrane. This is also true for cholesterol present in bilayers of small unilamellar phosphatidylcholine or phosphatidylserine vesicles made by ultrasonication. The data presented here on intestinal brush-border membrane are in good agreement with results reported on other biological membranes, e.g., from erythrocytes and vesicular stomatitis virus, but are somewhat different from those on rat intestinal brush-border membrane. Our results on phospholipid bilayers agree well with published work on model membranes. From the work presented we conclude that, with our present understanding, cholesterol oxidase can hardly be used to probe the distribution of cholesterol in biological membranes. A prerequisite for using the enzyme successfully as such a probe would be the understanding of the factors controlling the interaction of the enzyme with its substrate cholesterol. The question under which conditions cholesterol oxidase could be useful for probing the distribution and preferred location of cholesterol in biological membranes is discussed.

Introduction

The oxidation of cholesterol in human erythrocyte membranes in the presence of externally added cholesterol oxidase has been studied in detail [1–3]. In the unperturbed erythrocyte membrane there is no enzyme-induced oxidation of cholesterol. Oxidation is, however, induced by detergent disruption or by pretreatment of the erythrocyte membrane with phospholipase C [2]. In sealed ghosts, oxidation is induced if the medium is hypotonic or if the inner surface of the ghosts is exposed to the enzyme [1,3]. Similarly, the cholesterol in the intact membrane of vesicular stomatitis virus is resistent to cholesterol oxidase unless detergent is added or the membrane is treated with phospholipase C [2,4] or the membrane is enriched with exogenous cholesterol [18].

The possibility of using cholesterol oxidase to probe the distribution of cholesterol between the two bilayer halves of the brush-border membrane, and possibly other membranes, is intriguing. We have studied the oxidation of cholesterol by cholesterol oxidase in brush-border vesicles and have compared this with the oxidation of choles-

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Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; PA, phosphatidic acid; SM, sphingomyelin; SUV, small unilamellar vesicles; CTAB, cetyltrimethylammonium bromide; SDS, sodium dodecyl sulphate.

terol in single bilayer vesicles made of different phospholipids and of the lipids extracted from brush-border membrane. The results show that cholesterol present in intact brush-border membrane vesicles or lipid bilayers is not oxidized under standard conditions as used by us. Cholesterol oxidation takes place at appreciable rates only if the membrane is disrupted or solubilized by the addition of sufficient detergent.

Materials and Methods

Egg phosphatidylcholine and bovine spinal cord phosphatidylserine were purchased from Lipid Products (South Nutfield, U.K.), Δ^4-cholestenone from Serva (Heidelberg, F.R.G.), cholesterol and Cholesterol Merckotest (iodide method using cholesterol oxidase from *Nocardia* sp.) from Merck (Darmstadt, F.R.G.). Cholesterol oxidase (from *Nocardia erythropolis*) was obtained from Boehringer (Mannheim, F.R.G.). Both enzymes gave similar results. Triton X-100 was obtained from Sigma, 1-*O*-*n*-octyl β-D-glucopyranoside (octyl glucoside) from Boehringer, sodium dodecyl sulphate from BDH (Poole, U.K.), sodium cholate and cetyltrimethylammonium bromide from Merck. These detergents were used without further purification, their purity being given in the catalogues as over 99%.

Brush-border vesicles were prepared from rabbit small intestine (obtained from freshly slaughtered farm animals) that had been stored at -50°C essentially according to Hauser et al. [6]. The isolation buffer, referred to as the buffer, was 5 mM Hepes-NaOH (pH 7.6)/0.3 M D-mannitol/5 mM EGTA and the precipitation step was carried out with $MgCl_2$ (15 mM) instead of $CaCl_2$ [7,8]. The pellet of brush-border vesicles was resuspended in the buffer and the suspension was frozen and stored at -35°C until required. Total lipids were extracted from brush-border membrane according to Radin [9].

Cholesterol oxidase (EC 1.1.3.6) catalyzes the reaction

$$\text{cholesterol} + O_2 \rightarrow \Delta^4\text{-cholestenone} + H_2O_2$$

The progress of the reaction was followed by determining either H_2O_2 or cholestenone formed. When the production of H_2O_2 was measured, a linear relationship was found between H_2O_2 produced and the concentration of brush-border vesicles only if the brush-border vesicles were heat-denatured before the addition of Triton X-100 and cholesterol oxidase. For the same undenatured brush-border vesicles, using the same conditions, there was always less measurable H_2O_2 production and, moreover, the deviation from linearity increased with increasing brush-border vesicle concentration. In contrast, cholestenone production was found to be linear with brush-border vesicle concentration and the same whether or not the vesicles were denatured. It would seem, therefore, that brush-border vesicles contain enzyme(s) that utilize H_2O_2. For the determination of H_2O_2, aliquots of the reaction mixture were removed and diluted with 0.35 ml of the buffer, heated for 1 min at 100°C and centrifuged (12000 × *g* for 3 min). To 0.3 ml of the supernatant were added 0.3 ml of the Merckotest colour reagent; the mixture was incubated for 2 h and the absorbance at 390 nm was measured. The molar absorption was 13650 cm^{-1} and 13370 cm^{-1} for cholesterol added from a 10 mM stock solution in ethanol and 5% Triton X-100, respectively. Δ^4-Cholestenone production was determined directly by following the increase in absorbance at 240 nm. The molar absorption of cholestenone in methanol was determined as 15700 cm^{-1}. Unless specifically stated, all incubations and assays were done at room temperature (20 ± 2°C). Initial rates of cholesterol oxidation (as summarized in Tables I–III) were derived from the slope of the time-course of cholesterol oxidation (cf. Figs. 1 and 2A) at $t \rightarrow 0$.

For preparation of lipid vesicles, a solution of the lipid in choloroform/methanol (2 : 1, v/v) was rotary evaporated and dried under high vacuum. The lipid was suspended in the buffer at 10 mg · ml^{-1} and the dispersion was sonicated for 45 min (50% on/off cycle) with a 4 mm tip sonifier (Branson Sonic Power Co., Danbury, U.S.A.), under N_2 and cooling with ice/water. The vesicle suspension was then centrifuged for 5 min at 12000 × *g*.

Protein determination was done using the Bio-Rad protein assay (Bio-Rad Laboratories, Glattbrugg, Switzerland) with bovine serum albumin as the standard [10].

Results

Cholesterol was readily oxidized by cholesterol oxidase if it was contained in mixed micelles consisting of egg PC and an excess of Triton X-100 (Table I). In contrast, in other mixed micelles, in which Triton X-100 was replaced by either octyl glucoside (up to 15 mM), sodium cholate (up to 1.5 mM), SDS (up to 1.5 mM) or CTAB (up to 15 mM), the rate of cholesterol oxidation was insignificant. Subsequent addition of 1.5 mM Triton X-100 to the mixed micelles containing octyl glucoside or sodium cholate activated the enzyme and the rates of cholesterol oxidation measured then were similar to that observed with cholesterol/PC/Triton X-100 micelles (Table I). This indicates that octyl glucoside and sodium cholate did not denature the enzyme to any significant extent. In contrast, subsequent addition of Triton X-100 to mixed micelles containing either CTAB or SDS induced no increase in the oxidation rate of cholesterol. This is probably due to the interaction of these charged detergents with the enzyme, producing unfolding and inactivation.

The accessibility of cholesterol in phospholipid bilayers of SUV to oxidation by cholesterol oxidase is shown in Fig. 1. Sonicated PC/cholesterol (Fig. 1A) and ox-brain PS/cholesterol dispersions (Fig. 1B) were incubated at 20°C with cholesterol oxidase in the absence (open symbols) and presence (closed symbols) of 1.5 mM Triton X-100. The amount of detergent was sufficient to solubilize the SUV to mixed micelles as evident from the transition of the slightly turbid phospholipid dispersion to an optically clear solution. Both dispersion shown in Fig. 1 behaved similarly. In the absence of Triton X-100 less than about 10% of

TABLE I

OXIDATION OF CHOLESTEROL BY CHOLESTEROL OXIDASE AT 20°C IN MIXED MICELLES CONSISTING OF CHOLESTEROL, EGG PHOSPHATIDYLCHOLINE AND EXCESS OF DETERGENT

The incubation mixture contained 0.03 $U \cdot ml^{-1}$ of cholesterol oxidase from *N. erythropolis*. The values in parentheses are the initial rates of oxidation measured after the addition of 1.5 mM Triton X-100.

Mixed micelle	Initial rate of oxidation ($\% \cdot min^{-1}$)
Cholesterol/PC/Triton X-100 (75 μM/150 μM/1.5 mM)	12.5
Cholesterol/PC/octyl glucoside (75 μM/150 μM/15 mM)	< 0.1 (11.5)
Cholesterol/PC/sodium cholate (75 μM/150 μM/1.5 mM)	0.06 (8.0)
Cholesterol/PC/CTAB (75 μM/150 μM/15 mM)	< 0.1 (< 0.1)
Cholesterol/PC/SDS (75 μM/150 μM/1.5 mM)	< 0.1 (< 0.1)

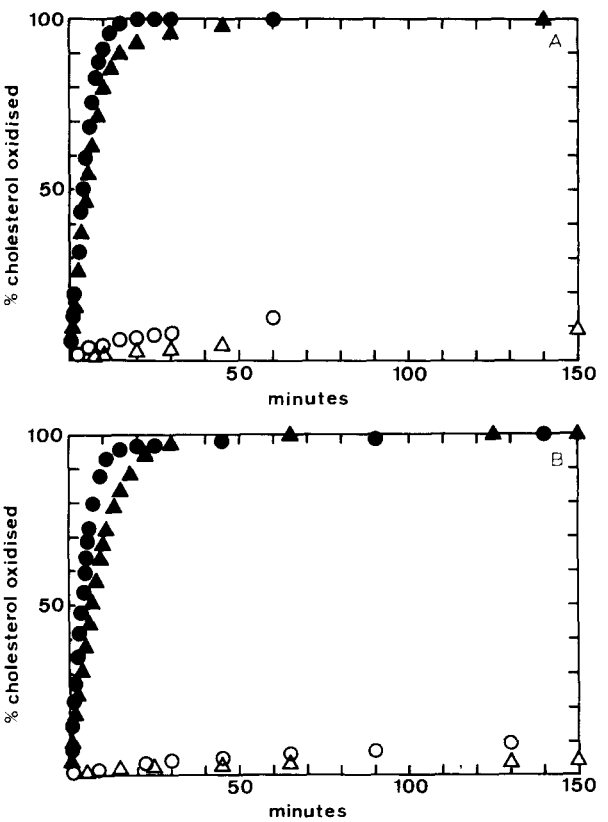

Fig. 1. Time-course of the oxidation of cholesterol by cholesterol oxidase. SUV of egg PC and cholesterol (A) or PS and cholesterol (B) were prepared by ultrasonication. The phospholipid concentration was either 0.032 mM (○, ●) or 0.113 mM (△, ▲) and the cholesterol concentration was 0.075 mM. The phospholipid bilayers at 0.032 mM can be assumed to be saturated with cholesterol. The phospholipid/cholesterol vesicles were incubated with cholesterol oxidase (0.03 $U \cdot ml^{-1}$ from *Nocardia* sp. and *Nocardia erythropolis*) at 20°C in the absence (○, △) or in the presence of 1.5 mM Triton X-100 (●, ▲). The reaction was followed by determining the absorbance at 240 nm of Δ^4-cholestenone formed during the reaction. Each point represents the average of two or three measurements.

the cholesterol was oxidized in 150 min. In contrast, in the presence of 1.5 mM Triton X-100, the initial rate of cholesterol oxidation was increased by a factor of 80–100. Initial oxidation rates are summarized in Table II. For comparison, SUV made from the lipids extracted from brush-border membranes were incubated with cholesterol oxidase. The initial rate of cholesterol oxidation was negligible in the absence of detergent. In the presence of 1.5 mM Triton X-100, it was increased similarly to those measured for the other phospholipid dispersions shown in Table II.

The accessibility of cholesterol in intact brush-border vesicle membranes to oxidation by cholesterol oxidase is shown in Fig. 2A. In the absence of Triton X-100 (open symbols), as with

TABLE II

OXIDATION OF CHOLESTEROL IN BILAYERS AND MICELLES BY CHOLESTEROL OXIDASE

The cholesterol was present in phospholipid bilayers of SUV made by sonication. The initial rate of oxidation is the initial slope (at $t \rightarrow 0$) of the time-course of cholesterol oxidation presented in Fig. 1. The values derived from the work of Patzer et al. [2] represent lower limits. Initial rates of oxidation were calculated from the amount oxidized in 240 min assuming a linear rate of oxidation.

Phospholipid bilayer or micelle	Concentration of detergent	Temperature (°C)	Cholesterol oxidase concentration ($U \cdot ml^{-1}$)	Initial rate of oxidation ($\%min^{-1}$)	Ref.
PC [c]/cholesterol (0.113/0.075 mM)	–	20	0.03	0.1	t.w. [a]
	Triton X-100 (1.5 mM)	20	0.03	11	t.w.
PC (0.032 mM) saturated with cholesterol	–	20	0.03	0.16	t.w.
	Triton X-100 (1.5 mM)	20	0.03	13	t.w.
PS/cholesterol (0.113/0.075 mM)	–	20	0.03	0.10	t.w.
	Triton X-100 (1.5 mM)	20	0.03	8.3	t.w.
PS (0.032 mM) saturated with cholesterol	–	20	0.03	0.17	t.w.
	Triton X-100 (1.5 mM)	20	0.03	14	t.w.
PC/cholesterol (0.5/0.5 mM)	–	37	0.025	0.01	2
PC/PE/cholesterol (0.25/0.25/0.5 mM)	–	37	0.025	0.04	2
PC/PA/cholesterol (0.47/0.025/0.5 mM)	–	37	0.025	0.04	2
PC/PA/cholesterol (0.4/0.1/0.5 mM)	–	37	0.025	0.09	2
PS/cholesterol (0.5/0.5 mM)	–	37	0.025	1.25 [b]	2
SM/cholesterol (0.5/0.5 mM)	–	37	0.025	0.01	2

[a] This work.

[b] This value was calculated from the work of Patzer et al. [2] showing that 98% of the cholesterol was oxidized in 4 h. The calculation assumes that the time taken for the reaction to go to completion (4 h) equals six half-lifetimes.

[c] PC and PE were from egg yolk.

small unilamellar phospholipid vesicles, the rate of cholesterol oxidation was very slow (cf. Table III). In the presence of 1.5 mM Triton X-100 (closed symbols) the rate of cholesterol oxidation was increased by a factor of 10^3 or more. A semilogarithmic plot of the fraction of cholesterol remaining against time was not linear, but biphasic (Fig. 2B), indicating that the reaction does not follow pseudo-first-order kinetics. The Triton X-100 concentration was sufficiently high to induce proteolysis of actin in the presence of trypsin [11]. The cytoskeletal protein actin has been shown to be located on the inner or cytoplasmic side of the brush-border vesicle membrane [12] and was used here as a marker. Its accessibility to proteolysis by trypsin indicated the lytic action of Triton X-100.

TABLE III

OXIDATION OF CHOLESTEROL IN BIOLOGICAL MEMBRANES BY CHOLESTEROL OXIDASE

Biological membrane	Concentration of detergent	Temperature (°C)	Concentration of cholesterol oxidase ($U \cdot ml^{-1}$)	Initial rate of oxidation ($\% \cdot min^{-1}$)	Ref.
Brush-border vesicles	–	20	0.05	0.05	t.w.[a]
	CTAB (40 mM)	20	0.05	1.1–2	t.w.
	–	20	0.53	0.05	t.w.
	–	20	0.03	0.01	t.w.
	Triton X-100 (1.5 mM)	20	0.03	10	t.w.
	–	20	0.50	0.04	t.w.
	Triton X-100 (1.5 mM)	20	0.50	150	t.w.
	–	37	0.35	0.1	t.w.
	CTAB (40 mM)	37	0.35	2	t.w.
Brush-border vesicles	–	37	0.5	1.2[c]	5
				0.2[c]	5
	Triton X-100 (25 mM)	37	0.5	30	5
Vesicular stomatitis virus	–	37	0.025	0.01	2
	taurodeoxycholate (5.7 mM)	37	0.025	~1.3[b]	2
Erythrocytes intact cells	–	37	0.025	0.01	2
	taurodeoxycholate (5.7 mM)	37	0.025	~1.3[b]	2
intact cells	–	37	1	0.0	3
unsealed red ghosts	–	37	20	60	14
unsealed red ghosts	–	37	1	3	3
resealed red ghosts	–	37	1	0.09	3
resealed white ghosts	–	37	1	0.30	3
resealed ghosts	–	37	0.025	0.01	2
resealed ghosts	–	37	20	0	1
leaky ghosts	–	37	20	25	1

[a] This work.

[b] These values are approximations, probably representing lower limits. The oxidation was found to go to completion in about 4 h; the calculated values given in the table were obtained by assuming that 4 h equals six half-lifetimes.

[c] In the absence of detergent, Bloj and Zilversmit [5] found the oxidation of cholesterol in the presence of cholesterol oxidase to be biphasic; the two numbers given in the table were derived from the sloopes of a semilogarithmic plot (Fig. 5 in ref. 5) similar to that shown in Fig. 2B and refer to the fast and slow rate of cholesterol oxidation.

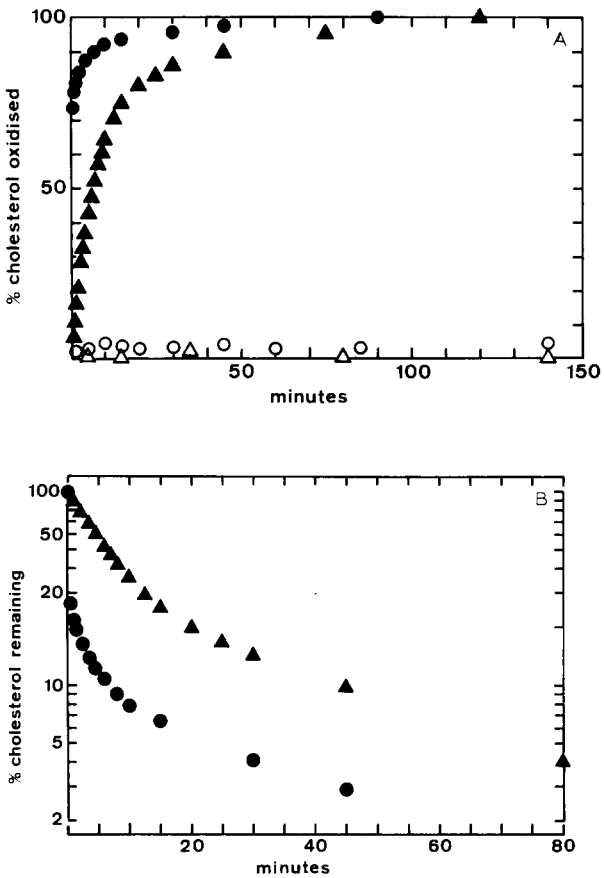

Fig. 2. (A) Time-course of the oxidation of cholesterol of brush-border membrane in the presence of cholesterol oxidase. Brush-border vesicles at 0.13 mg protein/ml buffer were incubated with 0.03 $U \cdot ml^{-1}$ (△, ▲) or 0.50 $U \cdot ml^{-1}$ (○, ●) cholesterol oxidase (either from *Nocardia* sp. or *Nocardia erythropolis*) in the absence (△, ○) or presence of 1.5 mM Triton X-100 (▲, ●). The progress of the reaction was followed by determining Δ^4-cholestenone production from the increase in absorbance at 240 nm. Each point represents the average of two or three measurements. (B) Kinetic analysis of the time-course shown in (A). The data in the presence of 1.5 mM Triton X-100 are plotted according to a pseudo-first-order reaction. If the oxidation of cholesterol were to follow pseudo-first-order kinetics, then $\ln X = -kt + \text{constant}$, where X is the fraction (expressed as %) of cholesterol remaining in brush-border membrane after time t.

In the presence of lytic concentrations of CTAB [11], the rate of oxidation of cholesterol was also increased; however, the increase, by a factor of about 20, was much smaller than that observed with Triton X-100 (Table III).

Discussion

One of the main conclusions of the work presented here is that cholesterol present in unperturbed bilayer membranes is a poor substrate for cholesterol oxidase. This is true for unperturbed phospholipid bilayers, both isoelectric and negatively charged, indicating that the surface charge cannot be critical. It is also true for intact biological membranes. Cholesterol becomes susceptible to oxidation by cholesterol oxidase in the presence of detergents. The quantity of detergent required was such as to perturb or disrupt the integrity of the membrane structure. Triton X-100 is apparently more effective than CTAB. This differential effect could be due to specific detergent-protein interactions; the positively charged CTAB possibly leads to a partial denaturation of the enzyme and in turn to a loss of activity. In general, the results presented here (Figs. 1 and 2 and Tables II and III) are in good agreement with data in the literature. Our results on brush-border membrane appear to be in agreement with those obtained with other intact or unperturbed membranes. The low values for the rate of cholesterol oxidation measured in brush-border membrane in the absence of detergent, particularly those at 20°C, were similar to those reported for intact erythrocytes [2,3] and vesicular stomatitis virus [2] (Table III). As shown in this table, the low values are also similar to those measured in resealed erythrocyte ghosts [1–3]. In contrast, the values reported for leaky ghosts [1,3,14] are significantly higher and comparable to the rates measured in brush-border membrane in the presence of detergent. Our results on brush-border membrane are, however, different from those reported by Bloj and Zilversmit [5]. Under comparable conditions, i.e., at 37°C and in the absence of detergent, the rate of cholesterol oxidation we measured ($0.1\% \cdot min^{-1}$) was of the same order of magnitude as the slow rate of cholesterol oxidation ($0.2\% \cdot min^{-1}$) reported by these authors. However, we failed to detect the fast initial rate of oxidation of at least $1.2\% \cdot min^{-1}$ reported by Bloj and Zilversmit [5] (cf. Table III). In our experiments, biphasic kinetics, as reported by these authors, were observed only in the presence of detergent (Fig. 2B). Bloj and Zilversmit [5] observed biphasic kinetics in the

absence of detergent, which were interpreted in terms of a heterogeneous distribution of cholesterol. By extrapolating the slow phase to zero time it was concluded [5] that 30% of cholesterol in brush-border membrane is accessible to the enzyme, while 70% is shielded from enzymatic attack. The proportion not accessible was proposed to be associated with membrane protein rather than to reflect the outside-inside distribution of cholesterol. Since in our hands intact brush-border membranes were not susceptible to oxidation by the enzyme, no conclusions regarding the distribution of cholesterol can be drawn from our experiments. The reason for this discrepancy is not clear at present. One possible explanation could be that Bloj and Zilversmit [5] used brush-border membrane isolated from the small intestine of cholesterol-fed rabbits. Such a treatment could result in cholesterol-enriched plasma membranes. It was shown both for membranes of red blood cells [3] and vesicular stomatitis virus that cholesterol enrichment with exogenous cholesterol rendered the membrane cholesterol (endogenous and exogenous) accessible to cholesterol oxidase.

Our results with SUV are also in good agreement with data in the literature (cf. Table II). The exceptions are the results obtained with SUV of PS and cholesterol (Table II) reported by Patzer et al. [2] and with SUV made of egg PC and 50 mol% cholesterol [18]. In both these cases, appreciable oxidation rates were observed in the absence of detergent. The reason for the discrepancy between these and our results is not clear at present.

The second conclusion derived from our work is that, given our present understanding of the interaction between cholesterol oxidase and cholesterol, the enzyme can hardly be used to probe the distribution and location of cholesterol in lipid bilayers or biological membranes. Marked differential susceptibility of cholesterol to oxidation by cholesterol oxidase has been reported before [1–3] and the data presented here confirm these results. The interaction between the cholesterol oxidase and its substrate cholesterol apparently depends in a subtle and profound way on the local environment of the substrate. As long as we do not understand the environmental factors controlling the activity of this enzyme, it cannot be used to probe cholesterol distribution in membranes.

The following brief consideration is pertinent to the question of the usefulness of cholesterol oxidase as a structural probe. Under which conditions would cholesterol oxidase be a useful probe for determining either the distribution of cholesterol between the two halves of the membrane or, possibly, its lateral distribution within one half of the membrane? Certain experimental conditions preclude the use of cholesterol oxidase as such a probe, even if the problem of controlling the activity of the enzyme in intact biological membranes was solved. It has been shown that interaction between lipid and protein can result in a lateral phase separation of the lipid, giving rise to an annulus of preferred lipid surrounding a particular protein [15–17]. It is now agreed that annular lipids exchange rapidly with the bulk lipids with frequencies $10^4 < \nu < 10^8\ s^{-1}$. If the rate of exchange of cholesterol between different environments is faster than the rate of cholesterol oxidation, monophasic kinetics will be observed. In this case, cholesterol oxidase will not give information on the lateral distribution of cholesterol. As the exchange rates observed between lipids in different environments are usually fast on the NMR timescale, it is highly unlikely that cholesterol oxidase will be useful for probing the lateral distribution of membrane cholesterol. Similar kinetic considerations are relevant when cholesterol oxidase is used for probing the transverse distribution of cholesterol between the two bilayer halves. Information on this type of distribution can be obtained only if the rate of cholesterol oxidation exceeds the rate of transmembrane movement, or flip-flop, of cholesterol. For instance, Lange et al. [14] showed that cholesterol could be oxidized in erythrocytes in which the cholesterol content had been artificially increased. Under this condition, all the cholesterol was oxidized in an apparently monophasic reaction with a half-time of 10 s. The authors concluded that the half time of cholesterol flip-flop in this membrane must be less than 3 s.

These simple considerations clearly show that the relative rates of the molecular motion of cholesterol and of the cholesterol oxidation are important and may restrict the use of cholesterol oxidase in probing the interaction and distribution of cholesterol in membranes. From the work presented here it is clear that future studies should be

addressed to the question of which factors control the interaction of the enzyme with cholesterol in membranes. This information is required if cholesterol oxidase is to be used as a probe of cholesterol distribution in biological membranes.

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