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THE REACTIVITY OF HUMAN ERYTHROCYTE MEMBRANE CHOLESTEROL WITH A CHOLESTEROL OXIDASE

MELVIN H. GOTTLIEB

National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Md. 20014 (U.S.A.)

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Summary

Cholesterol oxidase (EC 1.1.3.6, *Brevibacterium* sp.), which catalyzes the reaction: cholesterol + O_2 → Δ4-cholestenone + H_2O_2, has no effect on the cholesterol of intact (human) erythrocytes and of "resealed" ghosts, when it is present only outside these ghosts. The cholesterol of "leaky" ghosts, of "resealed" ghosts with enzyme trapped within, and of "inside-out" vesicles, was completely oxidized. This pattern indicates that the inner (cytoplasmic) membrane surface must be exposed to the enzyme for the reaction to occur, and that outer surface cholesterol only becomes reactive after the membrane has been degraded by the oxidation of inner surface cholesterol. The enzymatic oxidations followed monotonic first-order kinetics, and hence gave no evidence to support the two states of cholesterol in the membrane that had been postulated earlier from studies on the plasma lipoprotein extraction of cholesterol from the membrane.

Introduction

Little has been firmly established about the state of cholesterol in the erythrocyte membrane, although this substance constitutes 30% of the membrane lipid. An autoradiography study indicates that cholesterol is distributed in a belt around the convex portion of the cell [1], while an electron microscope study suggests that it is uniformly distributed throughout the membrane in small molecular groups [2]. The recent analyses of freeze fracture surfaces [3] points to an asymmetric distribution between the inner and outer membrane surfaces. The exchange of cholesterol between erythrocytes and plasma lipoproteins [4] shows that at least some is at the outer membrane surface, and the related extraction by plasma lipoproteins [5] suggests that it is present in two binding states.

The actions of various enzymes on the phospholipids of erythrocyte membranes have given information on the distribution of the major phospholipids between the inner and outer membrane surfaces [6] and also on the lateral surface pressure at the outer half of the membrane bilayer [7]. This present study of the action of the enzyme cholesterol oxidase on intact human erythrocytes, and several types of membrane ghost preparations, was undertaken to obtain information on the distribution of cholesterol between the inner and outer membrane surfaces, and to look for further evidence of the suggested two binding states.

The reaction catalyzed by the enzyme is [8]:

$$\text{Cholesterol} + O_2 \rightarrow \Delta 4\text{-cholestenone} + H_2O_2 \qquad (1)$$

Materials and Methods

Cholesterol oxidase (EC 1.1.3.6, *Brevibacterium* sp.) was used as obtained from Beckman Instruments, Inc., Carlsbad, Calif. Sodium dodecyl sulfate gel electrophoresis [9] showed only two bands, one of which contained about 75% of the protein and corresponded to a molecular weight of 50 000—60 000, and the other corresponding to a molecular weight of about 12 000. According to the supplier (personal communication) no detergent was used in the preparation or purification of the material. The particular enzyme sample used had an activity of 15.2 I.U./mg solid and 30 I.U./mg protein.

Fresh blood was collected and washed as described [5]. "Leaky" ghosts were prepared by hemolyzing cells in 40 volumes of cold (0—5°C) 5 mmol sodium phosphate, pH 8.0 (unless otherwise specified) and washing three times with this buffer. Ghosts thus washed do not reseal [10]. "Resealed" ghosts were similarly hemolyzed, but immediately after the hemolysis were incubated in phosphate-buffered saline (0.15 M NaCl, 10 mmol sodium phosphate to pH 7.4) at 37°C for 1 h to reseal [11].

"Inside-out" vesicles were prepared as described by Steck [10]. The three separate preparations studied contained 83, 78 and 85% "sealed, inside-out" vesicles, by the sialic acid method.

Analytical determinations. Cells and ghosts were extracted with isopropanol/chloroform [12]. Cholesterol and Δ4-cholestenone were both analyzed, using gas chromatography with cholestane as internal standard. Duplicate determinations agreed to within 2%. Mass balance computations indicated that Δ4-cholestenone accounted for about 95% of the reaction product.

Except for kinetic studies, the cells and membrane preparations were separated from the enzyme-containing solution by centrifuging before the extraction. Those preparations reported as not reacting showed no Δ4-cholestenone in either the sedimented or supernatant portions with this precaution. Direct extraction of the suspensions resulted in the oxidation of about 10% of the cholesterol, presumably by the rapid action, on the isopropanol-swollen membrane, of enzyme that had not yet been denatured by the isopropanol.

In kinetic studies, aliquots of the ghost suspensions were added directly into stirred isopropanol, and the amount of cholesterol oxidized during the extraction was estimated by extrapolation of the kinetic curve back to $t = 0$; typically

the correction amounted to 10% of the cholesterol. Duplicate kinetic runs gave first-order (vide-infra) reaction half-times that agreed within about 10%.

A N_2-filled glove box was utilized for those experiments carried out in the absence of O_2; enzyme solutions and ghost suspensions were stirred separately for 0.5 h in the N_2 atmosphere before mixing.

Results

Intact erythrocytes. The cholesterol of intact cells was unaffected by exposure, for 1 h, to an enzyme concentration of 20 I.U./ml, a concentration at which the first-order reaction half-time for "leaky" ghosts is about 5 min (vide-infra). However, despite the absence of cholesterol oxidation, hemoglobin analysis of the supernatant solution (by absorption at 540 nm), in three experiments, showed that 4—8% of the cells had hemolyzed, while only about half this hemolysis occurred in the absence of enzyme. Cells depleted of 30% of their cholesterol by extraction with preincubated plasma as described [5] behaved similarly to untreated cells with regard to both their unreactivity with the enzyme and the hemolysis.

Ghosts. Table I demonstrates the marked differences between the reactivities of "leaky" and "resealed" ghost cholesterol. For this experiment erythrocytes were hemolyzed in dilute solutions of several compositions, and the ghosts from each hemolyzing solution divided into portions to be maintained in the "leaky" state and to be "resealed". As shown in Table I, all of the "leaky" ghost cholesterol was oxidized, while none from the "resealed" ghost was. Microscopic examination showed that the "leaky" ghosts became fragmented as a result of the enzyme treatment, and that the "resealed"ghosts were unaffected.

Contrary to Eqn. 1, no H_2O_2 was detected, using the *o*-dianisidine method [13], during the oxidation of the "leaky ghost" cholesterol. The absence of H_2O_2 was presumably a result of the catalase and peroxidases in the membrane [14].

Further results with the two ghost types, and also with "inside-out" vesicles, follow.

TABLE I

COMPARISON OF REACTIVITIES OF "LEAKY" AND "RESEALED" GHOSTS

Reaction conditions: 50 μg/ml ghost cholesterol, 20 I.U./ml cholesterol oxidase, 37°C, 1 h.

Composition of hemolyzing solution	Ghost type	Percent conversion cholesterol to Δ4-cholestenone
50 mmol NaCl, 5 mmol sodium phosphate (pH 7.4)	"Leaky"	100
	"Resealed"	0
5 mmol NaCl, 5 mmol sodium phosphate (pH 7.4)	"Leaky"	100
	"Resealed"	0
5 mmol sodium phosphate (pH 7.4)	"Leaky"	100
	"Resealed"	0
5 mmol sodium phosphate (pH 8.0)	"Leaky"	100
	"Resealed"	0

"Leaky ghosts". To test whether the reactivity of the cholesterol of "leaky ghosts" was a secondary result of extraneous alterations of the inner surface of the membrane by the enzyme, the kinetics of the reaction of "leaky" ghosts that had been preincubated with the enzyme (in the absence of O_2, so as to forestall the cholesterol oxidation) was compared with that of ghosts from the same preparation that had not been preincubated. The reactivity was found not to be affected by the preliminary non-oxidizing, incubation with enzyme.

Monotonic first-order kinetics were found with all "leaky" ghost preparations. Fig. 1 shows typical course of the oxidation, Table II gives the averages of duplicate determinations of $t_{1/2}$, the first-order reaction half-times, under a range of conditions for four nominally similar ghost preparations. For comparison, results on cholesterol dispersed in a surfactant solution are also included. In all cases, the reaction was followed to at least 80% completion.

"Resealed" ghosts. The different reactivities of the "resealed" ghosts, and the "leaky" ghosts suggest that the cholesterol oxidase only reacts with cholesterol when it has access to the inner surface of the membrane. To test this, the reactivity of "resealed" ghosts was examined with enzyme present only in the solution enclosed by the ghosts. This was accomplished by (in the absence of O_2) adding enzyme immediately after the hemolysis step, then resealing and washing the "resealed" ghosts twice with phosphate-buffered saline. As one control, measurements were similarly made on "resealed" ghosts with enzyme present both inside and outside of the ghost, accomplished by preparing ghosts as above, and adding enzyme again after the wash steps. A second control involved measurements made with enzyme only outside the "resealed" ghost (as in Table I), accomplished by not adding any enzyme until after the resealing and washes. (Lower enzyme concentrations were used in these experiments to minimize oxidation by residual oxygen during resealing and washing procedures.) As shown in Table III (for one of two experiments run with similar results) membrane cholesterol of "resealed" ghosts was significantly oxidized

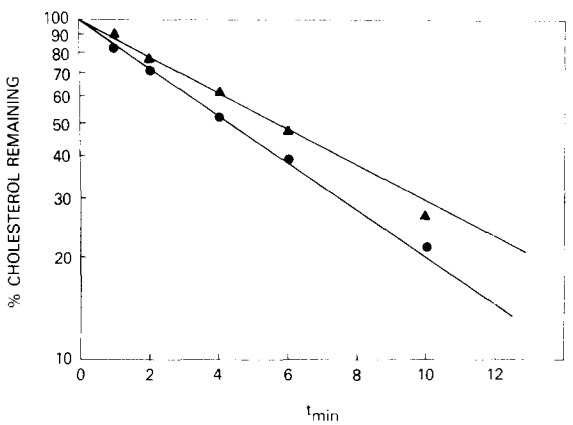

Fig. 1. Kinetics of reaction (37° C) of: ●, ordinary ghosts, 50 μg/ml ghost cholesterol, 20 I.U./ml cholesterol oxidase, and ▲, ghosts depleted of 30% of their cholesterol [5], 54 μg/ml ghost cholesterol, 20 I.U./ml cholesterol oxidase. The difference between the reaction rates is not significantly different from that between nominally similar ghost preparations (see Table II).

TABLE II

FIRST-ORDER REACTION HALF-TIMES, $t_{1/2}$, FOR "LEAKY" GHOSTS UNDER VARIOUS CONDITIONS

T = 37°C, 0.15 M NaCl, pH 7.4.

Ghost concentration (μg cholesterol per ml)	Enzyme concentration (I.U./ml)	$t_{1/2}$ (min)
11	20	2.4
22	20	2.0
54	20	2.8
50	2	41.1
50	5	18.2
50	20	3.1
52	20	4.6
52 *	20	5.0
54 **	20	6.1
Surfactant dispersed cholesterol, 50 μg/ml ***	0.1	4.6

* In an O_2 atmosphere, with solutions presaturated with O_2.

** Prepared from cells depleted of 30% of their cholesterol by extraction with preincubated plasma [5].

*** Prepared by dissolving 100 mg cholesterol in 5.0 ml warm ethanol and 6.0 ml Surfal (P.L. Biochemicals, Milwaukee, Wisc.) and diluting to 100 ml with phosphate-buffered saline.

when, and only when, the enzyme was in contact with the inner membrane surface.

"Inside-out" versicles. The previous experiments predict that the cholesterol of "inside-out" vesicles, in which the normally cytoplasmic surface of the membrane is exposed to the solution, would react with the enzyme, even though these vesicles are known to be sealed [10]. Three different preparations of such vesicles exposed to enzyme concentrations of 20 I.U./ml, at concentrations of 46—58 μg vesicle cholesterol per ml, did indeed react. Monotonic first-order kinetics were followed and $t_{1/2}$ values were similar to those of "leaky" ghosts shown in Table II.

TABLE III

ACTION OF CHOLESTEROL OXIDASE IN CONTACT WITH PARTICULAR SURFACES OF "RESEALED" GHOSTS

Reaction conditions: 47 μg/ml ghost cholesterol, 0.2 I.U./ml enzyme. Reaction timed from time of exposure to air. Oxidation at t = 0 presumably represents that occurring during sealing, due to incomplete removal of O_2.

Location of enzyme	Reaction time (h)	Cholesterol oxidized (%)
Inside only	0	2
	1	81
	3	90
Outside only	0	2
	1	2
	3	5
Inside and outside	0	0
	1	78
	3	95

Discussion

The results clearly show that membrane cholesterol is only oxidized when the enzyme has access to the membrane inner surface, and that with this access all of the membrane cholesterol becomes oxidized. This behavior is not a result of the location of all the cholesterol at the inner surface of the membrane, since the evidence that at least some is at the outer surface [3—5] is strong. Rather, the results indicate that the outer surface cholesterol is initially unreactive with the enzyme, and only becomes reactive following oxidation of inner surface cholesterol. This reaction pattern parallels that of those phospholipases (phospholipase A_2 from pancreas and *Crotalus adamenteus*, phospholipase C from *Bacillus cereus*, and cabbage phospholipase D) which do not react with the phospholipids of intact erythrocytes, but completely hydrolyze those of leaky ghosts [6]. Obviously this type of behavior does not permit the determination of the distribution of the substrate between the inner and outer membrane surfaces. It remains for future work to find if a cholesterol oxidase from another source would act analogously to those phospholipases with which the distribution of the membrane phospholipids was determined, i.e would act upon outer surface cholesterol without destroying the membrane.

The non-reactivity of the outer surface cholesterol with the enzyme may be a result either of factors affecting cholesterol itself, or of interactions between the enzyme and the membrane outer surface. Possible factors in the former case are the steric blockage of cholesterol from the enzyme, and an excessive lateral film pressure [7]; possible factors in the latter case are the presence of groups inhibitory to the enzyme, and the adsorption of the enzyme in a configuration that prevents its binding of cholesterol.

The oxidation of outer surface cholesterol that accompanies the reaction of inner surface cholesterol probably reflects a degradation of the membrane, and may be related to the fragmentation that was microscopically observed. The substitution of the reaction product, Δ4-cholestenone, for the cholesterol in the membrane, and the reaction of membrane components with H_2O_2 produced by the cholesterol oxidase reaction, are two possible causes of the membrane degradation. The monotonic time course (Fig. 1) of the enzymatic oxidation indicates that the membrane degradation, and reaction of outer surface cholesterol, occur rapidly following the oxidation of inner surface cholesterol.

The actual rates of the enzymatic oxidation are shown in Table II. Lines 1—3 show that $t_{1/2}$ is independent of the cholesterol concentration in the ghost suspension (at constant enzyme concentration), as would be expected from the first-order kinetics. Lines 4—6 show that $t_{1/2}$ is at least approximately inversely proportional to the enzyme concentration (at a given cholesterol concentration), indicating that the ratio of enzyme to cholesterol is below that at which saturation effects appear. Additional data would be required to test whether the rate per enzyme unit does indeed increase with higher enzyme activities as indicated by line 6. The absence of an effect of oxygen pressure on the rate (lines 7 and 8), at least in the range 0.2 atm (air) to 1.0 atm (pure O_2) indicates that the diffusion of O_2 to the enzyme · cholesterol complex is not a rate-determining step.

The most interesting feature of the data of Table II is that about 200 times

greater enzyme concentration is required to attain the same oxidation rate with the ghosts as with cholesterol dispersed in surfactant solution (line 9). The slowness of the enzymatic oxidation of inner membrane surface cholesterol could result from factors of the type suggested to be responsible for the unreactivity of outer surface cholesterol. Conceivably, the very same factors, acting to different extents, may be involved in both cases.

There is nothing in the kinetics of the enzymatic oxidation of erythrocyte membrane cholesterol to support the existence of cholesterol in two states of binding, as suggested earlier from lipoprotein extraction experiments [5], i.e. the oxidation kinetics are monophasic and the reaction rate of ghosts from which the presumed "unbound" cholesterol has been removed is similar to that of ordinary ghosts (Table II, Fig. 1). However, these findings do not necessarily mean that cholesterol is in a uniform state of binding, but only that the rate-determining step in the enzymatic oxidation is independent of the binding state.

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