The characterization and interconversion of three forms of cholesterol oxidase extracted from *Nocardia rhodochrous*

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The physical properties and the methods used for interconversion of three forms of cholesterol oxidase extracted from *Nocardia rhodochrous* by treatment with Triton X-100, trypsin or buffer alone provide evidence that these forms differ chiefly in the possession or absence of a hydrophobic anchor region connected by a trypsin-sensitive region. The hydrophobic domain normally integrates the enzyme into the cell membrane and confers amphipathic properties on the solubilized enzyme, causing adsorption to hydrophobic resins, aggregation when detergent is removed and formation of mixed micelles with detergent and cholesterol resulting in surface-dilution kinetic behaviour and activation by relatively high concentrations of water-miscible solvents. By contrast, only the enzymic fragment is extracted with trypsin and it behaves as a conventional soluble enzyme and does not aggregate or interact with hydrophobic resins, detergents or water-miscible solvents. As no phospholipid could be detected in the enzyme extracts, the detergent appears to act as a substitute for the cell-membrane lipids that would normally interact with the hydrophobic region. This cholesterol oxidase is an example of a prokaryotic enzyme possessing two closely associated catalytic functions, dehydrogenase and isomerase activities, and an anchoring function.

Cholesterol oxidases (EC 1.1.3.6), which catalyse the first step in cholesterol degradation to cholest-4-en-3-one, have been described from *Arthrobacter simplex* (Arima *et al.*, 1969), *Nocardia erythropolis* (Smith & Brooks, 1974), *Nocardia rhodochrous* (Buckland *et al.*, 1974), *Brevibacterium sterolicum* (Uwajima *et al.*, 1974), *Streptomyces griseocarneus* (Kerenyi *et al.*, 1975), *Streptomyces violascens* (Tomioka *et al.*, 1976), *Corynebacterium sterolicum* (Shirokane *et al.*, 1977) and *Actinomyces lavendulae* (Petrova *et al.*, 1979).

The cholesterol oxidase from *N. rhodochrous* is believed to be an intrinsic membrane-bound enzyme located on the outside of the cell. The enzyme may be extracted from cells by treatment with Triton X-100 or trypsin, or to a lesser extent with phosphate buffer, but not by mechanical cell disruption or by changes in pH or ionic strength (Cheetham *et al.*, 1980). The enzyme possesses

closely associated 3 β -hydroxy steroid oxidase and 3-oxo steroid Δ^4 – Δ^5 -isomerase activities (Cheetham, 1979a). The use of the *Nocardia* in the presence of high concentrations of organic solvents (Buckland *et al.*, 1975) and the immobilization of cholesterol oxidase and its use in aqueous/organic solvent emulsions has been described previously (Lilly *et al.*, 1975, 1976). The Triton-extracted enzyme has also been used for the clinical assay of human serum cholesterol (Richmond, 1973; Allain *et al.*, 1974).

In the present paper we have compared the physicochemical properties of the three forms of cholesterol oxidase, solubilized by Triton X-100, trypsin or buffer alone, and techniques for their interconversion have been developed. These properties are consistent with the presence or absence of a hydrophobic region in the Triton- and trypsin-extracted forms of the enzyme that interacts with detergents and other hydrophobic molecules and surfaces. This hydrophobic region is removed by the action of trypsin on intact cells or on the enzyme extracted with Triton X-100 or buffer. This information is important in relating the function of cholesterol oxidase to its structure and in evaluating

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the best techniques for the industrial use of this and similar enzymes.

Materials and methods

N. rhodochrous (N.C.I.B. 10554) was grown as previously described by Buckland *et al.* (1974) and the harvested cells were stored at -20°C before use. The wet to dry weight ratio of the paste was 4:1. The Triton- or buffer-extracted enzymes could be stored at 8°C for up to 6 months or frozen at -20°C for 1 year without loss of activity. However, the trypsin-extracted enzyme proved relatively unstable even when stored at 4°C ; 92% of the original activity was lost after only 2 days even when soya-bean trypsin inhibitor was present. The unpurified extracts were used, unless stated otherwise.

Sephadex G-200 and Blue Dextran were obtained from Pharmacia (GB), London W5, U.K., DEAE-cellulose (DE-52) was from Whatman Laboratory Products, Maidstone, Kent, U.K., and Amberlite XAD-2 was from Rohm and Haas, Croydon, U.K. Polyacrylamide, *NN'*-methylenebisacrylamide, ammonium persulphate, cytochrome *c*, Amberlite IRC-50, Triton X-100 (av. mol.wt. 643) and sodium dithionite were supplied by BDH Chemicals, Poole, Dorset, U.K. Mercaptoethanol and *NNN'*-tetramethylenediamine were from Koch-Light Laboratories, Colnbrook, Bucks., U.K., bovine serum albumin, horseradish peroxidase and sodium dodecyl sulphate were from Sigma Chemical Co., Kingston, Surrey, U.K., and Amido Black 10B was from Hopkin and Williams, Chadwell Heath, Essex, U.K. Merck t.l.c. plates (silica-gel 60 F-254) were obtained from Anderman and Co., East Molesey, U.K.

Diafiltration was performed by using a PM10 membrane and TCF10 ultra-filter (Amicon, High Wycombe, Bucks., U.K.). Chromatographic columns were from Pharmacia. A 12000 Vario-perpex peristaltic pump, a Uvicord 11 and a type-7000 Ultrorac fraction collector were from LKB-Producter A.B., Stockholm-Bromma 1, Sweden.

Protein determinations and the extraction and assay of the cholesterol oxidase activity of the whole cells or the various forms of extracted enzymes were performed as described by Cheetham *et al.* (1980). The rate of O_2 consumption during the formation of cholest-4-en-3-one was measured with an oxygen electrode (Rank Bros., Cambridge, U.K.), using the same reaction conditions as in the spectrophotometric assay. In each case tested the amount of O_2 consumed was equimolar with the amount of cholest-4-en-3-one produced.

The interconversion of the various forms of cholesterol oxidase was followed by measuring the changes in the detergent concentration required to

obtain the maximum enzyme activity. Triton-, buffer- and trypsin-extracted enzymes had optima at Triton X-100 concentrations of 0.15, 0.005 and 0% (v/v) respectively.

Triton X-100 was assayed by the method of Garewal (1973) and Amberlite XAD-2 was used to remove Triton X-100, as described by Cheetham (1979b). Polyacrylamide-gel electrophoresis was carried out by the method of Cooston & Loomis (1969), except that all the buffers contained 0.5% (v/v) Triton X-100. Samples contained 30–500 μg of protein/ml and gels were stained with 0.5% (w/v) Amido Black in 7% (v/v) acetic acid and destained in the same acetic acid solution. Organic solvent extracts, made by the method of Rouser *et al.* (1967), were chromatographed on silica-gel plates in chloroform/methanol/water (65:30:4, by vol.). After chromatography the plates were stained with iodine or Dittmer's reagent.

Chromatography on Sephadex G-200 columns (28 cm $\times$ 2.5 cm diam.) was done at 8°C with 3 ml samples of enzyme, eluting with buffer at a flow rate of 15 ml/h and collecting 2.5 ml fractions. Columns were calibrated with cytochrome *c*, bovine serum albumin, horseradish peroxidase and Blue Dextran (to mark the void volume).

Ion-exchange chromatography was done on columns (18 cm $\times$ 1 cm diam.), as described by Buckland *et al.* (1974), except that a 1.0 mM-potassium phosphate buffer, pH 7.0, containing 0.5% Triton was used.

Results

General properties

All three forms of cholesterol oxidase had the following common properties: (i) pH optima at pH 7.0 (Fig. 1); (ii) similar temperature-activity profiles with optima at 30°C (Fig. 2 shows the results for the Triton X-100-extracted enzyme); (iii) substrate specificities similar to that described by Smith & Brooks (1974) for the detergent-extracted enzyme; (iv) inhibition to the same extent by cholest-4-en-3-one with a K_1 of 50 mM; (v) complete inhibition by 10 mM- HgCl_2 .

The Triton-extracted enzyme contained one major and three minor component proteins when analysed by polyacrylamide-gel electrophoresis. The major band was identified as the cholesterol oxidase protein by incubating the gels with substrate, then slicing the gel and measuring at 240 nm the cholest-4-en-3-one produced by each homogenized slice. The Triton-extracted enzyme was reasonably stable over the temperature range 30 – 60°C , and lost only a quarter of its original activity after 60 h incubation at 50°C . At temperatures above 60°C the Triton-extracted enzyme was rapidly inactivated such that after 5 min

Fig. 1. The influence of pH on the activity of cholesterol oxidase extracted with Triton X-100 (O) or trypsin (Δ). The curve for enzyme extracted with buffer alone was similar to that for the detergent-extracted enzyme.

Fig. 2. The influence of temperature on the activity of cholesterol oxidase extracted with Triton X-100. The activities were obtained from initial rate measurements as described in the Materials and methods section.

incubation at 80°C only 30% of the original activity remained.

No spectrum characteristic of a coenzyme or prosthetic group was observed even after the enzyme had been flushed with N_2 , CO or O_2 , or after complete digestion by incubating with 0.10% (w/v) trypsin at 18°C for 5 days. A similar absence of a cofactor has been reported for the hydroxy steroid oxidoreductases from *Streptomyces griseocarneus* (Kerenyi *et al.*, 1975) and *Streptomyces violascens* (Arima *et al.*, 1969).

The effect of detergent and cholesterol concentrations on enzyme activity

The trypsin-, buffer- and Triton-extracted enzymes were maximally active with zero, 0.005 and 0.15% Triton X-100 respectively (Fig. 3), and they had K_m values for cholesterol of 55, 45 and $69 \pm 0.3 \mu M$ respectively, as compared with a value of $24 \mu M$ for the cell-bound cholesterol oxidase. When Triton X-100 concentrations higher and lower than 0.15% were used the Triton-extracted-enzyme activity was decreased, but in each case the maximum activity was recovered by adjusting the detergent concentration back to 0.15%. Similarly, the activity of the enzyme extracted with high detergent concentrations, such as 10% (Cheetham *et al.*, 1980), increased as the extract was diluted, reaching a maximum activity at 0.15% detergent.

The activity of the Triton-extracted enzyme was dependent on both the detergent and cholesterol concentrations used, and high detergent concentrations decreased the cholesterol concentration required to obtain maximum activity (Fig. 4). Such surface-dilution kinetic behaviour is indicative of the formation of mixed micelles, especially as Dixon

Fig. 3. The variation with Triton X-100 concentration of the activities of cholesterol oxidase extracted with buffer alone (□), Triton X-100 (O) or trypsin (Δ).

Fig. 4. The influence of cholesterol and Triton X-100 concentrations on the activity of Triton-extracted enzyme. The concentrations of Triton X-100 (% v/v) in the assays were: 0.15 (○); 0.3 (△); 0.5 (□); 0.75 (●); 1.0 (▲).

plots showed that the inhibition of cholesterol oxidase by excess Triton X-100 was not simple competitive or non-competitive inhibition. Thus 0.15% (v/v) Triton X-100 and 0.10 mM-cholesterol, a detergent/cholesterol molar ratio of 24, were the concentrations used for routine assays.

Gel filtration

Further evidence for the existence of mixed micelles was provided by gel filtration on Sephadex G-200 in the presence of 0.5% (v/v) detergent. The Triton-extracted enzyme was resolved into three distinct peaks (Fig. 5), with a recovery of 80% of the activity. As the enzyme preparation applied to the column contained some residual cholesterol, cholest-4-en-3-one was co-eluted with the enzyme peaks. All three peaks had molecular weights greater than either the molecular weight of a pure Triton X-100 micelle, which is 64 000–77 000 (Hechtman & Kachra, 1980), or the molecular weight of the enzymic fragment, the trypsin-extracted enzyme, which was found to be only 35 000 by chromatography in the absence of Triton X-100. The first Triton-extracted-enzyme peak, in particular, had a molecular weight greater than 200 000, being excluded from the gel. Chromatography of the Triton- or buffer-extracted enzymes in the absence of

Fig. 5. Sephadex G-200 chromatography of Triton-extracted enzyme. Extraction of enzyme, conditions of chromatography and assay of the eluate fractions are as given in the Materials and methods section.

detergent caused both enzymes to aggregate and to be eluted in the void volume.

Concentration effects

A similar precipitation of the Triton- or buffer-extracted enzymes occurred whenever detergent was removed and especially when simultaneous concentration took place during diafiltration or vacuum dialysis. The solutions rapidly became cloudy as the detergent was removed and the volume was reduced. An enzymically active precipitate could be recovered after centrifugation at 15 000 g for 1 h at 25°C in an MSE 18 centrifuge (r_{av} , 5.6 cm), leaving little enzyme in the supernatant. Full activity could be regained by re-diluting the precipitate in buffer containing 0.15% (v/v) detergent.

The effects of organic solvents

Propan-2-ol markedly affected the activities of the three forms of enzyme (Fig. 6). At propan-2-ol concentrations greater than 23% (v/v) an abrupt and severe loss in Triton-extracted enzyme activity occurred, although full cholesterol oxidase activity could be regained by diluting with buffer back to a propan-2-ol concentration of 23% (v/v). Very similar results were obtained when n-propanol, acetone, ethanol or methanol were used as the co-solvent and when the overall Triton X-100 concentration was maintained constant at 0.15% by compensatory increases in the concentration of

Fig. 6. The effect of propan-2-ol concentration on the activity of cholesterol oxidase

The activities of Triton-extracted enzyme (○), trypsin-extracted enzyme (△) and buffer-extracted enzyme (□) were assayed spectrophotometrically in the presence of increasing concentrations of propan-2-ol.

detergent in the buffer. The enhancement in activity can be partially explained because the molar absorption coefficient of cholest-4-en-3-one is 1.2-fold greater in propan-2-ol than in water. Also propan-2-ol may decrease the size of the micelles, thereby increasing the total micellar surface available for uptake of substrate, or increase the activating effects of the detergents or be creating an environment closer to that experienced by the membrane-bound enzyme (Butler, 1979). The loss of activity at high propan-2-ol concentrations may be due to disruption of the mixed micelles, because pure Triton X-100 micelles are completely disaggregated by water-miscible solvent concentrations greater than 25% (v/v) (Becher, 1965). Buffer-extracted-enzyme activity was enhanced by propan-2-ol concentrations of up to 5% (v/v) but was lost at higher concentrations (Fig. 6). Trypsin-extracted enzyme lost activity even at low propan-2-ol concentrations.

Adsorption to resins

Both Triton- and buffer-extracted enzymes adsorbed to the hydrophobic resin Amberlite IRC-50 at pH 7.0 and 18°C. Appreciable quantities of Triton X-100 were also adsorbed with the Triton-extracted enzyme. The trypsin-extracted enzyme was not adsorbed, even at 4°C, or in the presence of soya-bean trypsin inhibitor, although these conditions did not affect the adsorption of the Triton- and buffer-extracted enzymes, again indicating that the

trypsin-extracted enzyme lacks the hydrophobic character of the Triton- and buffer-extracted enzymes.

All three forms could adsorb to DEAE-cellulose or to DEAE-Sephadex A-50 under the same conditions. The Triton-extracted enzyme was eluted from DEAE-cellulose in one sharp peak at 12 mM-NaCl with a recovery of 85% and an increase in specific activity to 7.3 units/mg of protein. This activity electrophoresed as a single band on polyacrylamide gel with a mobility identical with that of the major protein band of the unpurified Triton-extracted enzyme.

Phospholipid analysis

Organic solvent extracts of Triton-, buffer- and trypsin-extracted enzymes were made and chromatographed with phospholipid standards on silica-gel plates. When the plates were stained with iodine or Dittmer's reagent no phospholipid could be detected in any of the enzyme extracts.

Interconversion

Interconversion was monitored by measuring the influence of Triton X-100 on the activity of the enzyme (Fig. 3). When Triton-extracted enzyme was treated with 0.1% (w/v) trypsin for 1 h at 18°C and pH 7.0 it was converted into a trypsin-extracted-enzyme-like form. Incubation for a further 130 min caused a 50% loss in activity. The conversion could be done with a trypsin concentration as low as 0.005% (w/v), provided that incubation was at the optimal temperature and pH, but was prevented by addition of soya-bean trypsin inhibitor. Replacement of trypsin with chymotrypsin (0.1% w/v) resulted in a 1.3-fold enhancement in cholesterol oxidase activity after 2 min, followed by a progressive loss of activity characterized by a half-life of 35 min. When pepsin (0.1%, w/v) replaced trypsin a 6% enhancement of cholesterol oxidase was obtained after 15–30 min incubation, followed by a loss of activity with a half-life of approx. 5 h.

Buffer-extracted enzyme showed similar behaviour. Conversion into a trypsin-extracted-enzyme-like form was completed after 45 min incubation with 0.1% (w/v) trypsin, accompanied by a 3.2-fold loss in activity. Again, half of this remaining activity was lost during a further 60 min incubation. When chymotrypsin or pepsin replaced trypsin the former caused no activation and the latter only a 6% enhancement in activity, whereas both eventually caused a complete loss of activity.

Triton-extracted enzyme could be converted into a buffer-extracted-enzyme-like form by removal of Triton X-100 by using Amberlite XAD-2, followed by removal of the residual monomeric detergent by dialysis (Cheetham, 1979b). The enzyme retained 85% of its original activity and was now maximally

active in detergent-free buffer. This buffer-extracted-enzyme-like form could then be reconverted into a Triton-extracted-enzyme-like form by adding Triton X-100 to the buffer-extracted enzyme to a concentration of 0.5% (v/v).

The buffer-extracted-enzyme-like form could also be converted into a trypsin-extracted-enzyme-like form by treatment with trypsin, as carried out for the Triton-extracted enzyme. After 1 h incubation, complete conversion had been achieved, giving a 1.55-fold increase in enzyme activity, but after a further 3 h incubation, activity had diminished to half of its original value. As trypsin treatment of the Triton-extracted enzyme and detergent-free Triton-extracted-enzyme preparations occurred at the same rate, it appears that incorporation of cholesterol oxidase into detergent micelles does not affect the rate of digestion.

Discussion

Despite the differing requirements for detergent the enzymic properties of the three solubilized forms of cholesterol oxidase are very similar, demonstrating that the buffer, Triton and trypsin extraction procedures solubilize basically the same enzyme. Those properties of the enzyme that do differ indicate that cholesterol oxidase has a bimodal amphipathic structure similar to that of many other integral membrane-bound proteins such as cytochrome b_5 (Ito & Sato, 1968), NADH:ferri-cytochrome b_5 oxidoreductase (Spatz & Strittmatter, 1973), γ -glutamyltransferase (Hugkey & Curthoys, 1976), β -lactamase (Simons *et al.*, 1978), and many intestinal and renal brush-border hydrolases (Louvard *et al.*, 1975; Booth *et al.*, 1979; Brunner *et al.*, 1979; Maroux & Louvard, 1976) such as the small-intestinal sucrase-isomaltase complex. These proteins are composed of structurally separate hydrophilic and hydrophobic domains linked by a proteinase-sensitive connecting region. The hydrophilic domain possesses the enzymic activities and the predominantly hydrophobic anchor domain is responsible for binding the whole enzyme to membranes.

Intact but not trypsin-treated cholesterol oxidase binds to *Nocardia* cells (Cheetham *et al.*, 1980), hydrophobic resins and detergent micelles, indicating that the hydrophobic region must be responsible for the interaction. Similar behaviour has been described for papain-treated sucrase-isomaltase (Brunner *et al.*, 1978) and cytochrome b_5 (Faucon *et al.*, 1976). Unlike many other intrinsic proteins such as glycophorin (Tomita & Marchesi, 1975) and HLA antigens (Springer & Strominger, 1976), cholesterol oxidase protrudes out of the membrane. The term 'stalked intrinsic membrane protein' has been proposed for this class of proteins. It has been

speculated that the anchor region is derived from a redundant 'signal' peptide (Frank *et al.*, 1978), or by fusion of the genes coding for the enzyme and a hydrophobic protein so as to form a multifunctional protein.

The influence of detergent concentration on the activities of the extracted enzymes gives a further insight into the nature of the enzyme. The trypsin-treated enzymes behave like conventional water-soluble enzymes and so do not require detergents to stimulate activity (Fig. 3). The buffer-extracted form of cholesterol oxidase is most active at Triton X-100 concentrations below the critical micelle concentration (Fig. 3). The hydrophobic region of each enzyme molecule may require a coating of detergent molecules to prevent the enzyme-enzyme interactions that lead to aggregation and consequent loss of activity. A similar dispersing effect of detergent has been described for the rat small-intestinal sucrase-isomaltase by Yamashiro & Gray (1970).

The Triton-extracted enzyme appears to act as a nucleus for detergent binding and requires micellar Triton X-100 for the full expression of enzyme activity. The detergent may have a direct activatory effect on the enzyme, but it is likely that the enhanced activity is due to the formation of mixed micelles in which local high concentrations of enzyme and substrate occur. The formation of cholesterol oxidase/detergent mixed micelles is also indicated because of the very high molecular weight of the Triton-extracted enzyme and the effect of water-miscible organic solvents on the enzyme activity. Confirmatory direct evidence could be obtained by using the nitrene probes developed for hydrophobic regions by Owen *et al.* (1980). Because of this mixed-micelle structure the detergent concentration affects enzyme activity, changing the cholesterol concentration required to saturate the enzyme (Fig. 4). The reduction of activity at high detergent concentrations would result from competition between mixed micelles and detergent micelles for enzyme and substrate, or from dilution of the concentrations of enzyme and substrate in the mixed micelles. When extracts with a high detergent concentration were diluted to give the optimum detergent concentration for activity, the enzyme/detergent ratio decreased but the substrate/detergent ratio increased in the assay mixture. Similar surface-dilution kinetics have been described for phospholipase A_2 and phosphatidylserine carboxylase (Dennis, 1973; Warner & Dennis, 1975). Bernath & Vieth (1972) have described a similar enhancement in lysozyme activity in the presence of surfactant concentrations in excess of the critical micelle concentration. They believed that activation of the enzyme was due to an orientation effect inside the mixed micelle. Bockerhoff & Jenson (1974) have pointed out that for an enzyme acting on a

water-insoluble substrate the K_m may represent the affinity of the enzyme for hydrophobic surfaces rather than simply the affinity for its substrate. Thus, rather than the bulk cholesterol concentration that is conventionally measured, the surface area provided by the substrate may be a more accurate estimate of the available substrate concentration.

We have demonstrated that cholesterol oxidase is an intrinsic membrane-bound enzyme, and that the properties of the enzyme vary with the method of extraction consistent with the presence or absence of a hydrophobic anchor region. *In vivo* the hydrophobic region is probably associated with cell membrane but, as no phospholipids are extracted with the enzyme, the role of the detergent appears to be as a substitute for the naturally occurring surfactants, especially as the variation in Triton-extracted-enzyme activity with the Triton concentration is very similar to the effect of phospholipid- or lipid-depleted cytochrome oxidase activity (Jost *et al.*, 1973). Cholesterol oxidase from *N. rhodochrous* is an example of a prokaryotic enzyme that possesses an anchor domain and two closely associated catalytic functions, the dehydrogenase and isomerase activities.

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