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Cholesterol oxidase from *Streptomyces hygroscopicus* and *Brevibacterium sterolicum*: effect of surfactants and organic solvents on activity

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We have studied systematically the effect of the non-ionic surfactants Thesit and Triton X-100, and of propan-2-ol (used as a substrate solubilizer) on the activity of the cholesterol oxidases from *Streptomyces hygroscopicus* (SCO) and *Brevibacterium sterolicum* (BCO). Low concentrations of Thesit lead to an activity increase with both enzymes; at higher surfactant concentrations the opposite effect occurs. Triton X-100 inactivates both enzymes at all concentrations. It is deduced that these surfactants exert their effects by interaction with the enzymes and not by affecting micellar phenomena. The effect of propan-2-ol on SCO, in contrast with that on BCO, depends on the buffer concentration (potassium phosphate). Other organic solvents induce results similar to those obtained with SCO and propan-2-ol. A significant difference between the two cholesterol oxidases emerges when stability is tested at 25 °C and in the presence of different concentrations of propan-2-ol: BCO activity is rapidly inactivated, whereas SCO still has 70% of the initial activity after 5 h in the presence of 30% propan-2-ol. From our results, SCO seems to be the catalyst of choice in comparison with BCO for the exploitation of cholesterol oxidases in biotechnology and applied biochemistry.

Introduction

Cholesterol oxidase (3β-hydroxysteroid oxidase, EC 1.1.3.6) is the first enzyme in the pathway of cholesterol degradation in a number of soil bacteria. This FAD-dependent oxidase catalyses both the oxidation of 3β-hydroxysteroids having a *trans* double bond at Δ^5–Δ^6 of the steroid ring, yielding H_2O_2 and the corresponding Δ^5-ketosteroid, and also the isomerization of the latter to the Δ^4-3-ketosteroid. Cholesterol oxidases have a fairly broad substrate specificity and can oxidize (with varying efficiency) many different 3β-hydroxysterols, the presence of a 3β-hydroxy group being essential for activity [1]. Cholesterol oxidases are a group of enzymes found in, or secreted by, various micro-organisms. Some of them, e.g. those from *Nocardia*, *Brevibacterium* and *Streptomyces*, are commercially available. Cholesterol oxidase is industrially important for applications in bioconversions and useful for the clinical determination of total or free serum cholesterol [2].

The three-dimensional structure of cholesterol oxidase from *Brevibacterium sterolicum* (containing non-covalently bound FAD) has been solved [3]: the enzyme's catalytic site is formed by a hydrophobic cavity where the sterol binds and interacts with the flavin [3,4]. In spite of the availability of this three-dimensional structure and the wide range of applications and interests, detailed characterizations of kinetic and physicochemical properties of cholesterol oxidase, namely those from *Streptomyces hygroscopicus* and from *B. sterolicum* (which contains covalently bound FAD), have appeared only recently in publications [5]. Both enzymes are monomeric (molecular mass 55 kDa), acidic (pI 4.4–5.1) and contain FAD as a prosthetic group. Marked differences between the two enzymes are the midpoint redox potentials and their reactivities towards sulphite [5]. These differences might be eventually related to the difference in cofactor binding between *S. hygroscopicus* and *B. sterolicum* cholesterol oxidase. In the first the FAD is bound non-covalently, whereas in *B. sterolicum* the flavin is linked to the protein backbone via an 8-histidinyl-FAD residue.

From the whole body of data on the activity of cholesterol oxidase from different sources, and measured under different experimental conditions, the fact emerges that the effect of organic solvents, surfactants and ionic strength is fundamental in the context of cholesterol oxidase catalysis [5–7]. Furthermore, enzyme activity can vary not only with the presence of a given surfactant but also with the

Abbreviations used: BCO, recombinant cholesterol oxidase from *Brevibacterium sterolicum*; CMC, critical micelle concentration; SCO, cholesterol oxidase from *Streptomyces hygroscopicus*; Thesit®, dodecylpoly(ethylene glycol ether)$_n$ (n = 9–10).

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ratio of surfactant to substrate and with the ionic strength of the medium. This emphasizes the need for standardization of these parameters for optimal activity. In this context, detailed kinetic studies with cholesterol oxidase suffer from the very low solubility of steroid substrate in aqueous media. Moreover, the strong tendency of cholesterol to self-associate in water affects its monomeric concentration and prevents an accurate evaluation of the catalytic parameters of cholesterol oxidase in this solvent [8]. The surfactants and solvents usually employed to solubilize the steroids affect not only the enzyme activity but also the micellar composition of the system. The catalytic-centre activity, determined with cholesterol as substrate, after a concomitant increase in propan-2-ol concentration [from 1% to 10% (v/v)] and a decrease in the buffer ionic strength (from 500 to 50 mM), decreased to one-quarter [5] and to two-fifths (L. Pollegioni, unpublished work) for cholesterol oxidase from *S. hygroscopicus* (SCO) and recombinant *B. sterolicum* cholesterol oxidase (BCO) respectively.

The present study is a detailed evaluation of the interrelations of organic solvents, surfactants, enzyme and substrate(s) at different ionic strengths on the basis of our previous data [5]. The scope of the study is to provide a basis for the understanding of these relationships and for the evaluation of possible sources of inaccuracy in assessments of enzyme activity. This might improve the usability of cholesterol oxidase in biotechnology as well as in clinical chemistry and pave the way for detailed kinetic studies of the catalytic mechanism.

Materials and methods

Materials

Cholesterol, dodecylpoly(ethylene glycol ether)$_n$ (Thesit™) and Triton X-100™ were from Roche Diagnostics. Rhodamine 6G was from Sigma. All other reagents were of the highest purity commercially available. Androstensulphate (androst-5-en-3β-ol-β17-sulphate) was a gift from Dr. Dale Edmondson (Emory University, Atlanta, GA, U.S.A.). Both SCO [9] and BCO [10], expressed in *Escherichia coli* cells and then purified, were obtained from Roche Diagnostics.

Enzymic activity

Cholesterol oxidase activity was assayed by using the following assays: (1) measuring the oxygen consumption polarographically with a Rank electrode (O_2-detection method); (2) following the production of 4-cholesten-3-one spectrophotometrically at 240 nm (ε_{240} 15500 $M^{-1} \cdot cm^{-1}$; ΔA_{240} method); (3) quantifying the H_2O_2 produced with the enzyme-coupled assay with the use of horseradish peroxidase (0.01 mg/ml) and *o*-dianisidine (0.16 mg/ml) (H_2O_2 –peroxidase method) followed at 440 nm (ε_{440} 13000 $M^{-1} \cdot cm^{-1}$) and 25 °C as described previously [5]. All the measurements were performed in 50 mM potassium phosphate buffer, pH 7.5, unless specified otherwise.

Absorption and fluorescence measurements

Absorption spectra were recorded with a Uvikon 860 or 930 spectrophotometer (Kontron Instruments) in potassium phosphate buffer, pH 7.5, at 25 °C. Anaerobic experiments were performed on samples prepared by alternate evacuation and flushing with O_2-free nitrogen in 1 ml cells equipped with side arms.

Enzyme stability

To determine the stability in solution of BCO and SCO, enzyme samples were incubated at 25 °C and at three different final protein concentrations (1, 0.1 and 0.01 mg/ml) in 50 mM potassium phosphate buffer, pH 7.5. To assess the effect of propan-2-ol on enzyme stability, BCO and SCO samples (0.1 mg protein/ml) were incubated at 25 °C in 50 mM potassium phosphate buffer, pH 7.5, in the presence of 0–80% (v/v) propan-2-ol. In both cases, aliquots were retrieved periodically and assayed for cholesterol oxidase activity by using the H_2O_2–peroxidase method.

Determination of critical micelle concentration (CMC) of cholesterol

The CMC of cholesterol was determined at 25 °C in 50 mM potassium phosphate buffer, pH 7.5, by using a dye binding technique with 24 μM Rhodamine 6G as indicator; ΔA_{525} was followed [11].

Results

Effects of surfactants

Thesit In our previous study on BCO and SCO, a screening of the factors influencing the solubilization of cholesterol oxidase substrates was attempted [5]. The presence of the non-ionic surfactant Thesit very effectively improved the availability of sterol substrates to cholesterol oxidase [5]. The effect of increasing concentrations of Thesit on the activity of SCO and BCO is shown in Figure 1. In these experiments the activity was measured at a low substrate concentration (0.1 mM cholesterol) to detect the cholesterol oxidase activity even at low concentration of detergent (starting from 0.02% Thesit). A small amount of propan-2-ol (0.4%) was also present in the assay mixture because it was necessary for optimal solubilization of the substrate. For both enzymes an increase in Thesit concentration was initially paralleled by a marked increase in activity, after which the attained activity level remained constant for BCO but SCO activity diminished.

To ascertain whether the enzyme activation at low detergent concentration was due to the formation of mixed

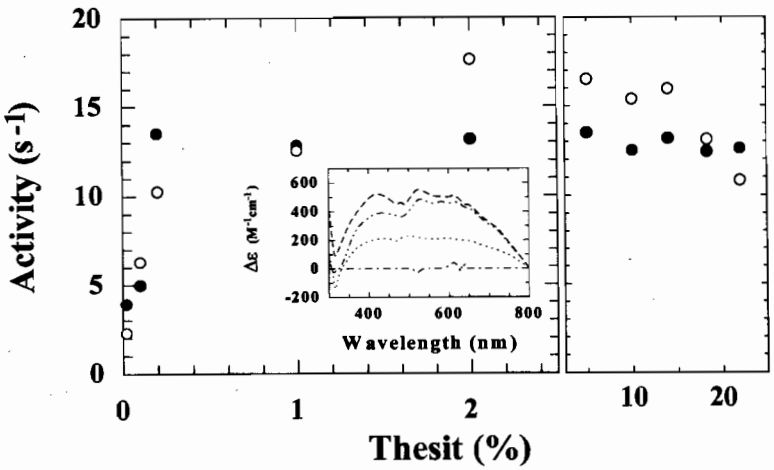

Figure 1 Dependence of SCO (○) and BCO (●) activity on the concentration of Thesit

Catalytic-centre activity was measured at 25 °C with 0.1 mM cholesterol as substrate in 50 mM potassium phosphate buffer, pH 7.5, in the presence of 0.4% (v/v) propan-2-ol, with the use of the H_2O_2–peroxidase method. Inset: effect of Thesit on the absorbance spectrum of SCO. The differential spectra were recorded at 25 °C with 25.2 μM SCO in 50 mM potassium phosphate buffer, pH 7.5, on the addition of 0% (dot–dashed line), 0.6% (dotted line), 6.7% (double-dot–dashed line), and 11.7% (broken line) Thesit (all final concentrations, v/v).

Table 1 Critical micelle concentration for cholesterol and effect of Thesit and propan-2-ol

Increasing concentrations of cholesterol or androstensulphate (in parenthesis), were added to solutions of 24 μM Rhodamine 6G in potassium phosphate buffer, pH 7.5, (KP_i) at 25 °C. CMC values were estimated by detecting ΔA_{525}, by the method of [11]. Concentrations of Thesit and propan-2-ol are expressed as percentages (v/v) .

Conditions	CMC (μM)
Water[a]	11
50 mM KP_i/1% Thesit	7
50 mM KP_i/0.2% Thesit/1% propan-2-ol	17
50 mM KP_i/1% Thesit/1% propan-2-ol	40
50 mM KP_i/1% Thesit/5% propan-2-ol	30
50 mM KP_i/1% Thesit/10% propan-2-ol	15 (205)
500 mM KP_i/1% Thesit	130
500 mM KP_i/1% Thesit/10% propan-2-ol	13

[a] Value taken from [19].

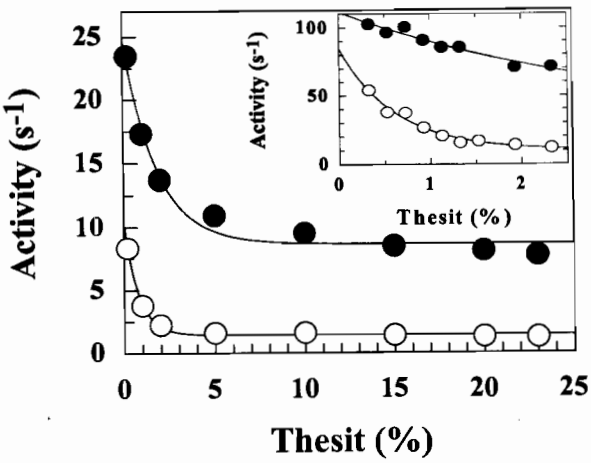

Figure 2 Dependence of SCO (○) and BCO (●) activity on the concentration of Thesit

Conditions were as described in the legend to Figure 1 except that 0.34 mM cholesterol was used as substrate. Inset: same conditions except in the presence of 500 mM potassium phosphate. Catalytic-centre activity is shown.

micelles of cholesterol with the detergent molecules [11], we determined the CMC for cholesterol at different Thesit concentrations and in the presence of different amounts of propan-2-ol (Table 1). At 50 mM potassium phosphate, the increase in cholesterol CMC given by the presence of Thesit was limited, always remaining in the micromolar range. In this context it should be noted that, at least at low potassium phosphate concentration, the cholesterol concentration used in the assay mixtures was in all cases above this range. The formation of mixed micelles of cholesterol and surfactant cannot be excluded [12]. In any event, the presence of non-inverted cholesterol micelles at the low propan-2-ol concentration used can be envisaged [the CMC of Thesit in water is 0.09–0.15 mM, corresponding to 0.5–0.9% (v/v) Thesit] [13]. It is known that the incorporation of substrate into micelles improves the utilization of substrate by cholesterol oxidase. However, the activation of both cholesterol oxidases at low Thesit concentration can most probably be assumed to follow the pattern observed in some enzymes whose catalytic activity, at a determined enzyme-to-surfactant ratio, is stimulated by 'soft surfactants' such as non-ionic surfactants and bile salts [14,15]. This suggests a specific interaction between surfactants and proteins in aqueous solutions [13].

Under the same experimental conditions as in Figure 1, but at higher substrate concentration (0.34 mM), the effect of increasing surfactant concentration on the activity was drastically different. Thus, as shown in Figure 2, a sharp

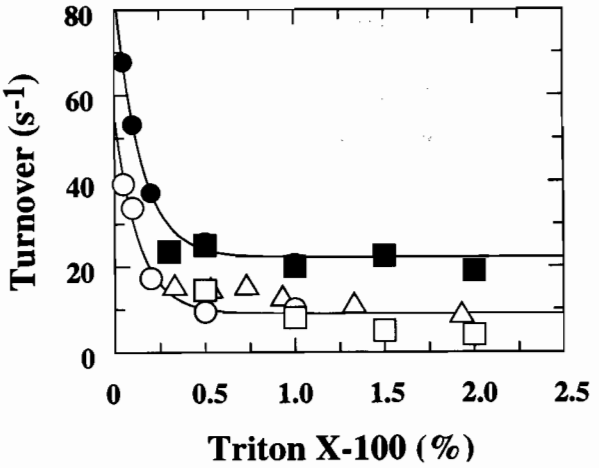

Figure 3 Dependence of SCO (□,○,△) and BCO (■,●,▲) activity on the concentration of Triton X-100

Conditions were as described in the legend to Figure 1 except that 500 mM potassium phosphate buffer and three different assays were used: the ΔA_{240} method (□,■), the H_2O_2–peroxidase method (○,●) and the O_2-detection (polarographic) method (△,▲).

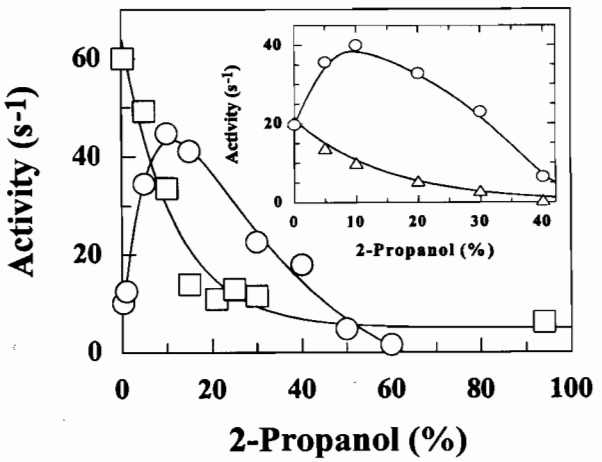

Figure 4 Dependence of SCO activity on the concentration of propan-2-ol

Conditions were as described in the legend to Figure 1 except that 50 mM (□) or 500 mM potassium phosphate buffer (○) and 0.4% (v/v) Thesit were used. Catalytic-centre activity was assessed with the H_2O_2–peroxidase assay, with 0.34 mM cholesterol as substrate. Inset: effect of propan-2-ol on SCO activity with 0.5 mM cholesterol (○) and 2 mM androstensulphate (△) as substrate, with the use of the ΔA_{240} method (conditions as described in the legend to Figure 4).

decrease as a function of Thesit concentration was observed at 50 and 500 mM potassium phosphate. Increasing the Thesit concentration also induced the same decrease in SCO activity when 1% or 10% (v/v) propan-2-ol was present in the assay medium (results not shown). The higher activity measured at 10% propan-2-ol agrees with the lower CMC value for cholesterol determined at this Thesit concentration (Table 1). The interaction between Thesit and the protein under these conditions also altered the visible portion of the absorbance spectrum of SCO as shown by differential spectroscopy (Figure 1, inset). Thus an increase in Thesit concentration from 0% to 11.7% (v/v) resulted in a broad increase in absorbance in the 350–800 nm range and in the appearance of an isosbestic point at 330 nm. From these effects an apparent interaction constant $K_d \approx 1\%$ Thesit can be estimated, corresponding to the concentration at which approx. 50% of the activity was found.

Triton X-100 As a comparison with Thesit, we studied the effect of a second non-ionic surfactant, Triton X-100, on the activity of SCO and BCO, with 0.1 mM cholesterol as substrate in three different assays (Figure 3). Because of the absorbance of Triton X-100 at 240 nm, detergent concentrations higher than 2% (v/v) could not be used in connection with the ΔA_{240} method. With all the assay methods, a decrease in activity was observed with increasing surfactant concentration; this result indicated the absence of specific effects of Triton X-100 on the assays itself. In fact, at 50 mM potassium phosphate, in the presence of 1% (v/v) Triton and 0.4% (v/v) propan-2-ol, there was also good agreement between the rates determined for both cholesterol oxidases with the two different assays: 47 compared with 38 s^{-1} for BCO and 6.8 compared with 6.4 s^{-1} for SCO by following the formation of 4-cholesten-3-one ($\Delta A_{240\,nm}$ assay) as well as that of H_2O_2. The marked decrease in activity as a function of Triton X-100 concentration observed for both cholesterol oxidases was similar to the results determined with Thesit at higher concentrations of cholesterol (see Figure 2). In general and for the purpose of solubilizing cholesterol, higher activity was found by using a low concentration (0.05%, v/v) of Triton X-100 than of Thesit.

Effect of organic solvents

Propan-2-ol The effect of propan-2-ol on SCO activity was studied at two potassium phosphate concentrations. The results (Figure 4) indicate a marked stimulation of activity in the presence of 500 mM potassium phosphate and at low propan-2-ol concentration, with maximal activity at approx. 10% (v/v) propan-2-ol. In contrast, a decrease in activity accompanied the increase in propan-2-ol concentration in 50 mM potassium phosphate (Figure 4). The latter result can be ascribed only partly to an increase in the apparent K_m for cholesterol [0.2, 0.6 and 2.4 mM at 0%, 1% and 10% (v/v) propan-2-ol respectively]; in fact the halving of the k_{cat} [126 s^{-1} at 1% (v/v) propan-2-ol and 58 s^{-1} at 10% (v/v) propan-2-ol] points to an effect on enzyme activity. However, a better utilization of sterol substrate probably

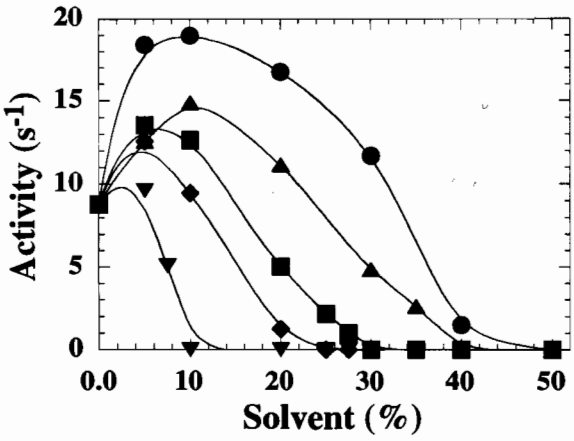

Figure 5 Effect of various solvents on the activity of BCO: propan-2-ol (●), ethanol (▲), propan-1-ol (■), acetonitrile (◆) and dioxan (▼)

Conditions were as described in the legend to Figure 1, with 1.25 mM cholesterol as substrate and with the use of the ΔA_{240} method.

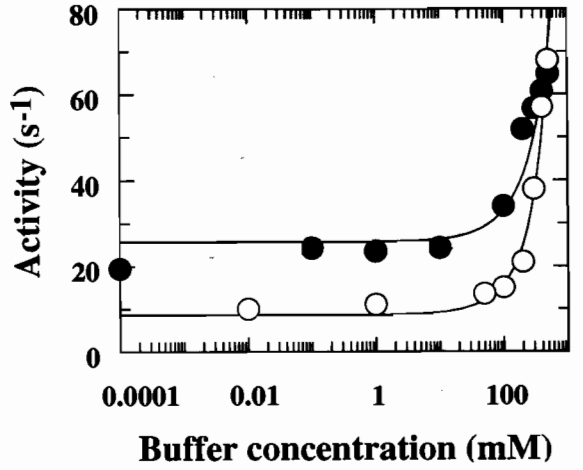

Figure 6 Dependence of BCO (●) and SCO (○) activity on potassium phosphate buffer concentration

Conditions were as described in the legend to Figure 1 in the presence of 0.4% (v/v) Thesit, with 1 mM cholesterol as substrate and with the use of the ΔA_{240} method.

occurred at higher potassium phosphate concentration in the presence of 10% (v/v) propan-2-ol because the presence of the alcohol decreased the CMC for cholesterol (Table 1). This is in agreement with results obtained previously for cholesterol oxidase from *Nocardia* [12]. Interestingly, at high ionic strength (500 mM potassium phosphate, ΔA_{240} assay), the activity of BCO was unaffected by variation of the alcohol concentration in the 1–10% (v/v) propan-2-ol concentration range (V_{max} 61±1.5 units/mg of protein; K^{chol}_m 0.15±0.04 mM). Furthermore, the dependence of SCO activity on propan-2-ol concentration varied with the nature of the substrate. As shown in Figure 4 (inset), the enzyme activity determined with cholesterol as substrate increased with increasing alcohol concentration up to 10% (paralleling the decrease in the CMC; Table 1), whereas with the more hydrophilic substrate androstensulphate an activity decrease always occurred. The formation of substrate micelles was probably less favoured with androstensulphate than with the more hydrophobic cholesterol, as also confirmed by the higher CMC found with the former (Table 1). Propan-2-ol perturbed the visible spectrum of SCO, producing a marked increase in absorbance below 380 nm, detectable up to 40% (v/v) alcohol concentration. At higher propan-2-ol concentrations the changes induced in the visible portion of the spectrum were most probably due to protein aggregation (results not shown).

The results suggest that the limited change in the dielectric constant given by the low concentration of propan-2-ol does not alone account for the solvent effect on enzyme activity. Depending on the alcohol presence, a change in some parameters of water could influence cholesterol oxidase activity, or a slight conformational change of the protein could occur [6,12,16].

Other solvents The effect of propan-1-ol, ethanol, dioxan and acetonitrile on the activity of BCO, with cholesterol as substrate, was also studied comparatively. A low concentration of each of these solvents in the assay mixture stimulated enzyme activity, although to different extents, as shown in Figure 5.

Effect of phosphate buffer concentration

The activities of BCO and SCO exhibited a marked increase when the buffer concentration reached the threshold of approx. 90 mM (Figure 6). These measurements were made with 1 mM cholesterol, a concentration higher than the CMC value (Table 1), i.e. a micellar system was constantly present in this case. The buffer solubility, and thus its concentration, decreased significantly, increasing the propan-2-ol concentration to more than 50% (results not shown). This fact might explain the rapid decrease in activity observed at high propan-2-ol concentration in the presence of 500 mM phosphate buffer, in addition to the more general denaturing effect (Figures 4 and 6).

Stability

The time course of the stability of SCO and BCO in solution was studied by incubating each enzyme at 25 °C in the presence of 50 mM potassium phosphate buffer, pH 7.5. Both cholesterol oxidases maintained more than 65% of initial activity after 24 h at 25 °C. The loss of activity was not influenced by the enzyme concentration in the incubation mixture in the 0.01–1 mg/ml protein range (results not

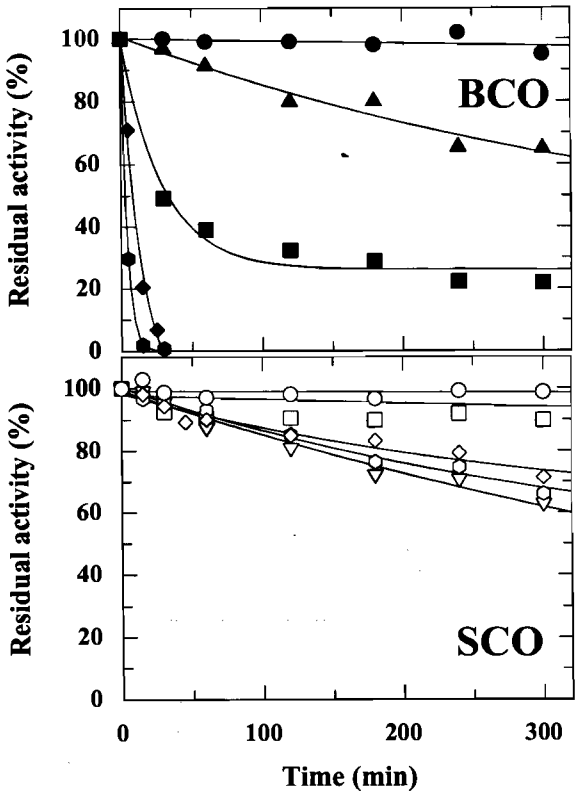

Figure 7 Stability of BCO and SCO, and the effect of propan-2-ol

The enzymes, 0.1 mg/ml protein in 50 mM potassium phosphate buffer, pH 7.5, were incubated at 25 °C at the following concentrations of propan-2-ol: ○,●, 0%; △,▲, 10%; □,■, 20%; ◇,◆, 30%; ⬡, ⬢, 40%; ▽, 60% (final concentrations, v/v). Residual activity was determined at 25 °C by using the H_2O_2–peroxidase method with 0.34 mM cholesterol as substrate.

shown). In addition, both cholesterol oxidases were completely stable for up to 300 min in the presence of 1% (v/v) propan-2-ol and 1% (v/v) Thesit as well as in the presence of 10% (v/v) propan-2-ol and 8% (v/v) Thesit (results not shown). We also investigated the stability of SCO and BCO in the presence of different concentrations of propan-2-ol. The effect of propan-2-ol in the final concentration range 0–60% (v/v) on the time-dependent activity of the two enzymes is shown in Figure 7. With BCO the enzyme was completely inactivated after only 30 min in the presence of 30% (v/v) propan-2-ol whereas SCO lost only approx. 30% of the initial activity under the same conditions after 300 min. Complete inactivation of SCO could be achieved only with 80% (v/v) propan-2-ol. The stability of both cholesterol oxidases was also investigated in the presence of organic solvents other than propan-2-ol in the incubation mixture. Both cholesterol oxidases exhibited a remarkable stability when incubated at 25 °C in the presence of 10% (v/v) acetone: only a 20% decrease in activity during 4 h of incubation was observed (results not shown). Furthermore, enzyme activity was not affected by ethylene glycol [up to 20% (v/v)] in 50 and 500 mM potassium phosphate buffer.

Discussion

The present study provides some basis for understanding the effects induced by organic solvents, by the two non-ionic surfactants Thesit and Triton X-100, by buffer concentration and by some combinations of the same on the activities of SCO and BCO. The use of surfactants to measure catalytic parameters with sterol-derived substrates is essential for solubilization of the latter. A marked effect of propan-2-ol, solvents and surfactants on the activity of *Nocardia* cholesterol oxidase has been also reported [6,12,17]. However, and as already noted by others [17], not all detergents are suitable for use with cholesterol oxidase: the presence of some surfactants in the assays causes a complete loss of enzyme activity. On the basis of our previous studies, Thesit, mainly in combination with propan-2-ol, has proved to be a suitable agent [5]. The stimulation of the activity of SCO and BCO at low concentrations of this surfactant can be ascribed not to the formation of mixed micelles of cholesterol with surfactant molecules (see [12]) but to a specific interaction between the detergent and the proteins, as also reported for other enzymes [13]. This follows from the observation that Triton X-100, a surfactant belonging to the same class of 'soft detergents' as Thesit, fails to induce activation and merely behaves as a denaturing agent. Analogously, a specific effect of bile salts on the activity of *Pseudomonas* cholesterol oxidase was observed previously [18,19], due mainly to an association of cholesterol oxidase and bile salt micelles, which increased enzyme activity without changing its affinity for the substrate. The effect of propan-2-ol, as well as that of other solvents, seems to be less specific. The initial increase in enzyme activity with increasing concentration of organic solvent can be rationalized by the incorporation of substrate into micelles; in this context, the different pattern observed with cholesterol as substrate in comparison with androstensulphate nicely supports this interpretation (Figure 4, inset).

A striking difference between the two cholesterol oxidases is the enzymes' (lack of) stability in the presence of increasing concentrations of propan-2-ol as documented in Figure 7. This is quite surprising, because the more susceptible BCO contains covalently bound FAD, which obviously cannot dissociate in the presence of solvent (cofactor dissociation resulting from effects induced by solvents or solutes is a common cause of inactivation of flavoproteins).

The present results therefore allow an optimization of the conditions for activity assay, for detection and for the conversion of cholesterol. These conditions seem to be low (phosphate) buffer concentration (less than 100 mM) and a

low concentration of Thesit and propan-2-ol [1% and 10% (v/v) respectively]. We also demonstrate that the use of 10% (v/v) propan-2-ol and 1% (v/v) Thesit in cholesterol standard solutions does not alter either the activity or the stability of BCO and SCO. Our results are in line with a study of the composition of assays for the enzymic determination of serum cholesterol [20], which indicated that SCO is the most suitable enzyme [compared with *Nocardia* and *Pseudomonas* cholesterol oxidases, and in the presence of 0.2% (v/v) Triton X-100 and 2 mM sodium cholate]. The conclusion in [20] was based on the economic cost and best stability of SCO. On the basis of the finding of the superior stability of SCO in the presence of the same organic solvents, this enzyme also seems to be the catalyst of choice compared with BCO. We consider this to be important information for the exploitation of cholesterol oxidases in biotechnology and applied biochemistry.

Acknowledgments

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References

1 Smith, A. G. and Brooks, C. J. W. (1976) J. Steroid Biochem. **7**, 705–713
2 Allain, C. C., Poon, L. S., Chan, C. G. S., Richmond, W. and Fu, P. C. (1974) Clin. Chem. **20**, 470–475
3 Vrielink, A., Lloyd, L. F. and Blow, D. M. (1991) J. Mol. Biol. **219**, 533–554
4 Vrielink, A., Brick, L. J. and Blow, D. M. (1993) Biochemistry **32**, 11507–11515
5 Gadda, G., Wels, G., Pollegioni, L., Zucchelli, S., Ambrosius, D., Pilone, M. S. and Ghisla, S. (1997) Eur. J. Biochem. **250**, 369–376
6 Khmelnitsky, Y. L., Hilhorst, R. and Veeger, C. (1998) Eur. J. Biochem. **176**, 265–271
7 Nishiya, Y., Yamashita, M., Murooka, Y., Fujii, I. and Hirayama, N. (1998) Protein Eng. **11**, 609–611
8 Tanford, C. (1973) The Hydrophobic Effect, p. 150, J. Wiley, New York
9 Wels, G. (1997) Ph.D. thesis, University of Konstanz
10 Jarsch, M. (1994) European Patent no. DE4342 012A1
11 Hsu, K. L. and Powell, G. L. (1975) Proc. Natl. Acad. Sci. U.S.A. **72**, 4729–4733
12 McGuinness, E. T., Brown, H. D., Chattopadhay, S. K. and Chen, F. (1978) Biochim. Biophys. Acta **530**, 247–257
13 Martinek, K., Levashov, A. V., Klyachko, N., Khmelnitsky, Y. L. and Berezin, I. V. (1986) Eur. J. Biochem. **155**, 453–468
14 McLonghlin, D. J., Shahied, I. I. and McQuarrie, R. (1978) Arch. Biochem. Biophys. **188**, 78–82
15 Hanozet, G. H., Pilone Simonetta, M., Barisio, D. and Guerritore, A. (1979) Arch. Biochem. Biophys. **196**, 46–53
16 Ke, T. and Klibanov, A. M. (1998) Biotechnol. Bioeng. **57**, 746–750
17 Miner-Williams, W. (1980) Clin. Chim. Acta **101**, 77–84
18 Cheillan, F., Lafont, H., Termine, E., Fernandez, F., Save, P. and Lesgards, G. (1989) Biochim. Biophys. Acta **999**, 233–238
19 Doukyo, N. and Aono, R. (1998) Applied Env. Microbiol. **64**, 1929–1932
20 Lolekha, P. H. and Teerajetkul, Y. (1996) J. Clin. Lab. Analysis **10**, 167–176