First publ. in: Flavins and flavoproteins 1999 : proceedings of the Thirteenth International Symposium, Konstanz, Germany, August 29 - September 4, 1999, pp. 627-630

Cholesterol oxidase from *Brevibacterium sterolicum* and *Streptomyces hygroscopicus*: a covalent FAD binding *vs.* a non-covalent one

Laura Motteran, Mirella S. Pilone and Loredano Pollegioni
Department of Structural and Functional Biology, University of Insubria, via J. H. Dunant 3, 21100 Varese, Italy
Sandro Ghisla
Faculty of Biology, University of Konstanz, P.O. Box 5560-M644, D-78434 Konstanz, Germany

Introduction

Cholesterol oxidase (CO, EC 1.1.3.6) is a flavoenzyme which catalyzes the first step in the pathway of cholesterol degradation in various microorganisms. CO is a bifunctional enzyme: it catalyzes the oxidation of β-hydroxysteroids and the isomerization of the produced Δ^5-ketosteroid to the Δ^4-3-ketosteroid. The three dimensional structure of CO from *Brevibacterium*, which contains non-covalently linked FAD, has been reported (1). Intriguingly, a second CO from a different strain of *Brevibacterium* contains a covalently linked 8-histidyl-FAD. We compare here two related COs, one from *S. hygroscopicus* (SCO) containing a freely dissociable cofactor, the other a recombinant protein from *B. sterolicum* (BCO) which contains covalently linked FAD and whose three-dimensional structure is close to completion (A.Vrielink, personal communication).

The redox and general properties of these two enzymes have been described recently. Marked differences between the two COs are the midpoint potentials and their reactivity towards sulfite (2). Further, we have studied a mutant form of BCO (H69A) obtained by site-directed mutagenesis, in which 8α-N1-histidyl-FAD bond cannot form. Although substantial advances have been made in recent years in understanding the mechanism of flavin attachment to protein, the role of the covalent link in the biological function is obscure (for a review see (3)). The aims of the present study is to assess the role of the FAD covalent link in CO by investigation of the stability and of some kinetic properties of these COs.

Materials and Methods

Cholesterol oxidase activity was assayed at 25 °C by different methods: Test 1 monitored the production of 4-cholesten-3-one spectrophotometrically at 240 nm (isomerization reaction); Test 2 monitored H_2O_2 production at 440 nm in enzyme-coupled assay with HRP (2); Test 3 was the enzyme monitored turnover (EMTN) method, in which enzyme and substrate were mixed in a stopped-flow instrument and the absorption change was monitored at ≈ 450 nm. Rapid reaction measurements and turnover experiments were carried out at 25 °C in 50 mM potassium phosphate

buffer, pH 7.5, in the presence of 1 % Thesit and 10 % 2-propanol (except where stated otherwise), in a thermostated stopped-flow spectrophotometer, provided of a diode array detector.

Results and Discussion

Spectral properties

Both SCO and BCO (wild-type and H69A) exhibit rather different absorption spectra in their oxidized states (λ_{max} = 467, 448 and 444 nm, respectively). Large differences are also evident among the two BCO proteins in the reduced state (λ_{max} = 362 and 406 nm for wild-type and H69A, respectively). A more substantial difference emerges with respect to the flavin semiquinone forms. Thus with H69A-BCO the red, anionic semiquinone is not significantly produced during photoreduction (≤ 5 % compared to ≈ 70 % for the wild-type enzyme) (Fig. 1).

Stability

The effect of organic solvents, surfactants and ionic strength is fundamental in the context of CO catalysis, since these factors affect not only the solubility of steroid substrate (which is extremely low in aqueous media) and the micellar composition of the system, but also the enzyme activity. In previous studies we have identified optimal conditions for an accurate and reproducible assay of CO activity (low phosphate buffer concentration, less than 100 mM, and low concentration of Thesit and 2-propanol, 1 % and 10 % respectively) (2, 4).

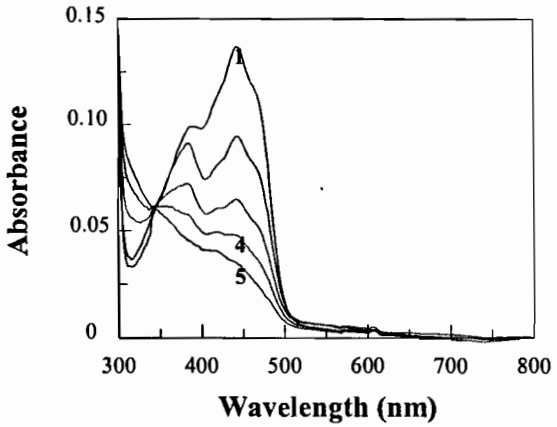

Figure 1: Photoreduction of H69A-BCO. 8.55 µM enzyme in 100 mM potassium phosphate buffer, pH 7.5, at 25 °C was made anaerobic and subsequently mixed with 0.5 µM 5-deaza-flavin (spectrum 1). The further spectra were recorded after 180 min (2), 220 min (3), 250 min (4) and 300 min (5) of light irradiation.

Surprisingly, a striking difference between the two COs emerges when stability is assessed at 25 °C using Test 2 and in the presence of different 2-propanol concentrations. At 1 % 2-propanol BCO and SCO show similar stability, at high alcohol concentration BCO, which contains covalently linked FAD, is more susceptible to denaturation. The H69A mutant, which has a freely dissociable FAD, shows (in)stability properties comparable to those of wild-type BCO.

Kinetic mechanism

The kinetic mechanism of BCO and SCO with cholesterol as substrate was studied by a combination of steady state and pre-steady state measurements (5). Under the optimal conditions for activity assay (see above), SCO and BCO show a similar rate of flavin reduction, although for the latter a 2-fold lower k_{cat} was determined (Table 1). A stronger decrease in catalytic activity is evident in the H69A mutant BCO: the lack of FAD-linkage resulted in an enzyme form showing a 200-fold reduction in k_{cat} and 3-fold decrease in K_m for cholesterol. Based on these results, BCO was proposed to work via a ping-pong (binary complex) mechanism whereas a ternary complex mechanism is active for SCO (for the latter, under specific experimental conditions of cholesterol oxidation, some rate constants are sufficiently small that the bimolecular term ϕ_{SO2} of the steady state equation is negligible giving a parallel lines pattern in the Lineweaver-Burk plot, see Table 1).

Table 1. Comparison of Steady State Coefficients and Reductive Half-Reaction Rate Constants for the Reaction of SCO, Wild-Type and H69A-BCO with Cholesterol as Substrate.

	Steady state			RHR	
	Lineweaver-Burk pattern	k_{cat} (s^{-1})	$K_{m,chol}$ (mM)	k_{red} (s^{-1})	$K_{d,app}$ (mM)
SCO (5)	≈ convergent	202	0.91	232	1.5
BCO wild-type (5)	parallel	105	0.87	235	0.16
BCO H69A	parallel	0.55	0.27	n.d.	

Experimental conditions: 100 mM potassium phosphate buffer, pH 7.5, 1 % Thesit and 10 % 2-propanol, at 25 °C.

The Δ5-6 → Δ4-5 isomerization reaction

The conversion of 5-cholesten-3-one, the assumed intermediate, into 4-cholesten-3-one, the final product is followed by monitoring the absorbance changes at 240 nm which accompany it (Test 1). This reaction was previously demonstrated to be

which accompany it (Test 1). This reaction was previously demonstrated to be efficiently catalyzed only by the oxidized form of the enzyme (the reduced form of SCO was approximately 2000-fold slower) (5). Comparison of the rates of 5-cholesten-3-one isomerization with those for the oxidation of cholesterol (see Table 1), showed that for neither enzyme was the Δ5-6 $\rightarrow$ Δ4-5 isomerization rate-limiting (the k_{cat} for the isomerization reaction is 670 s^{-1}, 200 s^{-1} and 60 s^{-1} for SCO, wild-type and H69A-BCO respectively).

Conclusions

Approximately 25 different enzymes have been reported to contain covalently bound flavins, in which the coenzyme can be attached to the protein moiety at the 8-CH_3 or the C6 atom of the isoalloxazine ring (3). Several COs, as the BCO we studied, belong to the former group. In CO, the loss of the covalent link of the cofactor affects the spectral properties and does not influence the substrate binding and protein stability. Unexpectedly, wild-type BCO (having the covalently attached FAD) shows a lower stability at high 2-propanol concentration compared to SCO. Thus, it is tempting to conclude that the FAD-linkage in BCO is an important factor in attaining the fully competent form of the enzyme and in the modulation of its redox properties. Comparison of the properties of COs having covalently and non-covalently bound FAD with their 3D-structure will considerably expand our knowledge on this flavoenzyme and on the role of flavin covalent attachment.

References

1. Li J., Vrielink A., Brick P., Blow D.M. (1993): Crystal structure of cholesterol oxidase complexed with a steroid substrate: implications for flavin adenine dependent alcohol oxidases. Biochemistry **32**, 11507-11515.

2. Gadda G., Wels G., Pollegioni L., Zucchelli S., Ambrosius D., Pilone M.S., Ghisla S. (1997): Characterization of cholesterol oxidase from *Streptomyces hygroscopicus* and *Brevibacterium sterolicum*. Eur. J. Biochem. **250**, 369-373.

3. Mewies M., McIntire W.S., Scrutton N.S. (1998): Covalent attachment of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) to enzymes: the current state of affairs. Protein Science **7**, 7-20.

4. Pollegioni L., Gadda G., Ambrosius D., Ghisla S., Pilone M.S. (1999): Cholesterol oxidase from *Streptomyces hygroscopicus* and *Brevibacterium sterolicum*: effect of surfactants and organic solvents on activity. Biotechnol. Appl. Biochem. **30**, 27-34.