Cholesterol Oxidase from *Brevibacterium sterolicum*

THE RELATIONSHIP BETWEEN COVALENT FLAVINYLATION AND REDOX PROPERTIES*

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***Brevibacterium sterolicum* possesses two forms of cholesterol oxidase, one containing noncovalently bound FAD, the second containing a FAD covalently linked to His[69] of the protein backbone. The functional role of the histidyl-FAD bond in the latter cholesterol oxidase was addressed by studying the properties of the H69A mutant in which the FAD is bound tightly, but not covalently, and by comparison with native enzyme. The mutant retains catalytic activity, but with a turnover rate decreased 35-fold; the isomerization step of the intermediate 3-ketosteroid to the final product is also preserved. Stabilization of the flavin semiquinone and binding of sulfite are markedly decreased, this correlates with a lower midpoint redox potential (−204 mV compared with −101 mV for wild-type). Reconstitution with 8-chloro-FAD led to a holoenzyme form of H69A cholesterol oxidase with a midpoint redox potential of −160 mV. In this enzyme form, flavin semiquinone is newly stabilized, and a 3.5-fold activity increase is observed, this mimicking the thermodynamic effects induced by the covalent flavin linkage. It is concluded that the flavin 8α-linkage to a (N1)histidine is a pivotal factor in the modulation of the redox properties of this cholesterol oxidase to increase its oxidative power.**

The flavoprotein cholesterol oxidase (CO)[1] (EC 1.1.3.6) is an alcohol dehydrogenase/oxidase that catalyzes the dehydrogenation of C(3)-OH of the cholestan system to yield the corresponding carbonyl product (Scheme 1). During the reductive half-reaction, the oxidized FAD accepts a hydride from the alcohol, and in the ensuing oxidative half-reaction, the reduced flavin transfers the redox equivalents to molecular oxygen, which is the final acceptor. COs are a group of enzymes isolated (and frequently secreted) from various microorganisms, they are characterized by a broad substrate specificity within the cholestan family. The first three-dimensional structure of a CO was that of the enzyme from *Brevibacterium sp.*, which con-

tains a noncovalently linked FAD (1); the structure of the CO from *Streptomyces hygroscopicus* (SCO) has recently been reported (2). Both enzymes possess the classical Rossman fold for dinucleotide binding found in many flavin-dependent oxidases (3) and belong to the glucose-methanol-choline oxidoreductase family (4).

A second CO was isolated from a strain reported to be a *Brevibacterium sterolicum* (BCO) (5). Intriguingly, it has a different primary sequence, it contains a FAD covalently linked to N(1) of His[69], and its three-dimensional structure belongs to a different family compared with the two COs mentioned above.[2] A similar situation was also reported in the two related enzymes D- and L-nicotinic acid oxidase from *Arthrobacter oxidans*: one contains covalently linked FAD and is specific for the substrate D-form (6); the second acts on the L-form and does not possess a covalent flavin. Some properties of the COs from *S. hygroscopicus* (containing noncovalently linked FAD) and from a recombinant protein from *B. sterolicum* (with covalently linked FAD) were described and compared recently (7). The effects of organic solvents, surfactants, and ionic strength on CO activity have been investigated (8). With only the exception of glucose oxidase, CO is the most widely used enzyme in clinical laboratories (for a recent and exhaustive review on CO applications in analytical chemistry, see Ref. 9).

BCO catalysis proceeds via a ping-pong mechanism, whereas SCO follows a sequential pathway (10). Although the two enzymes have similar rates of flavin reduction ($\sim$230 s^{-1} in the presence of 1% Thesit and 10% propan-2-ol), the turnover number at infinite oxygen and cholesterol concentrations is higher for SCO than for BCO (202 s^{-1} and 105 s^{-1}, respectively). Alcohols can be used as detergents and are useful for solubilizing steroids such as cholesterol. The effect of propan-2-ol on CO consists in an increase of enzyme activity with increasing concentration of the alcohol up to 10% (v/v) (8). This has been attributed to the effect of the alcohol on the availability of substrate that is incorporated into micelles and to the decrease of the critical micelle concentration for cholesterol (8). While SCO is slowly reduced by propan-2-ol under anaerobic conditions with 1.3 M propan-2-ol (half-time $\approx$86 min at 25 °C), BCO does not react under similar conditions (10). The isomerization reaction (Scheme 1) is efficiently catalyzed by the oxidized form of both enzymes and is never rate-limiting in catalysis. In the last few years, the reaction mechanism of CO has also received some attention (11), and studies based on directed mutagenesis have been conducted (12).

Up to now, more than 30 flavoenzymes have been reported to contain flavin covalently linked to a histidine, cysteine, or

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[1] The abbreviations used are: CO, cholesterol oxidase; SCO, cholesterol oxidase from *S. hygroscopicus*; BCO, recombinant cholesterol oxidase from *B. sterolicum* expressed in *E. coli*; cholesterol, 5-cholestene-3β-ol; EMTN, enzyme monitored turnover; EFl$_{ox}$, oxidized enzyme; EFl$_{seq}$, enzyme flavin semiquinone; EFl$_{red}$, reduced enzyme; 8Cl-FAD, 8-chloro-FAD.

[2] R. Coulombe, K. Q. Yue, S. Ghisla, and A. Vrielink, submitted for publication.

tyrosine residue of the polypeptide chain (6, 13). The BCO used in the present work contains covalent flavin and is structurally distinct from the previously studied COs because it belongs to the subfamily of vanillyl-alcohol oxidase, that contains a fold proposed to favor covalent flavinylation (14). Although the existence of covalent flavin-protein linkages has been known for many years, only recently has its mechanism of formation emerged and its functions been proposed (13): (i) it allows saturation of the active site with the cofactor (particularly favorable for flavoenzymes that are localized in a flavin-deficient environment); (ii) it modifies the redox properties; (iii) it modulates substrate specificity; (iv) it protects the coenzyme from modification (*i.e.* hydroxylation) and inactivation; (v) it facilitates the electron transfer to another coenzyme; and (vi) it induces structural benefits such as improved protein stability.

To elucidate the function of covalent flavin linkage in BCO, we have studied a mutant in which the exchange His^{69} → Ala prevents formation of the histidyl-FAD bond. This was also spurred on by the expected results of the study of the three-dimensional structure of BCO. Comparison of the structure of the present BCO with the crystal structure of the noncovalent CO from *B. sterolicum*, particularly in light of the data presented here, is expected to expand our understanding of the role of the flavin covalent attachment in flavoprotein oxidases.

EXPERIMENTAL PROCEDURES

Materials and Enzymes—Cholesterol and Thesit™ (dodecyl poly(ethyleneglycol ether)$_n$, $n = 9$–10) were purchased from Roche Molecular Biochemicals. All other reagents were of the highest commercially available purity. Wild-type and H69A recombinant *B. sterolicum* CO, from *Escherichia coli*, were obtained from Roche Molecular Biochemicals.

Enzymatic Activity—Cholesterol oxidase activity was assayed at 25 °C monitoring H_2O_2 production at 440 nm ($\epsilon_{440} = 13$ mM^{-1} cm^{-1}) in an enzyme-coupled assay with 10 μg/ml horseradish peroxidase and 16 mg/ml *o*-dianisidine as previously described (7, 8, 10). The isomerization reaction (Scheme 1) is followed by monitoring the production of 4-cholesten-3-one from 5-cholesten-3-one spectrophotometrically at 240 nm ($\Delta\epsilon_{240} = 15.5$ mM^{-1} cm^{-1}) due to the formation of two conjugated double bonds. Another procedure used to assay the CO activity was the enzyme-monitored turnover (EMTN) method of Gibson *et al.* (15), in which the enzyme was mixed anaerobically with substrate in a stopped-flow instrument and the absorption change was monitored at 446 nm to follow reaction progress. At this wavelength the conversion of oxidized into reduced, enzyme bound flavin is detected. All concentrations mentioned in these experiments are those after mixing, *i.e.* at 1:1 dilution. Rapid reactions were routinely recorded in the 300–700 nm wavelength range with an acquisition time of 0.8 ms/spectrum and resolution of 1 nm, as detailed in Ref. 10. Enzyme activity was assayed in 50 or 100 mM potassium phosphate buffer, pH 7.5, containing 1% (v/v) Thesit and 1% or 10% (v/v) propan-2-ol. EMTN data were analyzed using traces at 446 nm according to the method of Gibson *et al.* (15) and using KaleidaGraph™ (Synergy Software).

Absorption and Fluorescence Measurements—UV-visible absorption spectra were recorded with an Uvikon 930 spectrophotometer (Kontron Instruments) in 100 mM potassium phosphate buffer, pH 7.5, at 25 °C. Extinction coefficients were determined by measuring the change in absorbance by heat denaturation (boiling 5 min in the dark). An extinction coefficient of 11.3 mM^{-1} cm^{-1} at 445 nm for free FAD and one of 10.6 mM^{-1} cm^{-1} at 445 nm for free 8Cl-FAD (8-chloro-FAD) were used. Photoreduction experiments were carried out at 15 °C using a cuvette containing ≈7.5 μM enzyme, 5 mM EDTA, and 0.5 μM 5-deazaflavin which had been rendered anaerobic by subjecting it to alternative cycles of vacuum and O_2-free argon. The enzyme was photoirradiated (7, 16) and the progress of the reaction was followed spectrophotometrically. Flavin and protein fluorescence measurements were carried out in a Jasco FP-750 spectrofluorometer in 100 mM potassium phosphate buffer, pH 7.5, at 15 °C. Protein fluorescence emission was monitored at 340 nm ($\lambda_{exc} = 285$ nm) and flavin fluorescence emission at 530 nm ($\lambda_{exc} = 450$ nm), using an excitation slits = 5 nm and an emission slit = 10 nm. The fluorescence data were analyzed using the Spectra Analysis Program (Jasco Corp.).

The dissociation constant of the apoprotein-coenzyme complex was calculated using the Stinson equation (17) and assuming a simple 1:1 equilibrium (PF $\rightleftharpoons$ P+F),

$$1/(1 - a) = 1/K_d([L_0]/a - [E_0] \qquad \text{(Eq. 1)}$$

where a, fractional saturation of the total concentration of binding sites; K_d, dissociation constant of the enzyme-ligand complex; [L_0], total ligand concentration; and [E_0], total enzyme concentration.

Enzyme Stability—To determine the stability in solution of wild-type and H69A BCO, enzyme samples were incubated at 25 °C and at 0.1 mg/ml final protein concentration in 50 mM potassium phosphate buffer, pH 7.5. To assess the effect of propan-2-ol on enzyme stability, the samples (0.1 mg of protein/ml) were incubated in the presence of 0–60% (v/v) propan-2-ol. In all cases, aliquots were retrieved periodically and assayed for cholesterol oxidase activity using the H_2O_2 peroxidase method.

Redox Potentials—Redox potentials for the EFl_{ox}/EFl_{seq} and EFl_{seq}/EFl_{red} couples of CO were determined using the dye equilibration (18) method with the xanthine/xanthine oxidase reduction system at 15 °C (19). An anaerobic cuvette containing ≈12 μM enzyme, 0.2 mM xanthine, 5 μM benzyl viologen, and 1–10 μM of the appropriate dye was purged of oxygen, and the reaction was initiated by addition of 10 nM xanthine oxidase. The reaction was measured spectrophotometrically until completion, typically 3–4 h. Data were analyzed as described by Minnaert (18). The amount of oxidized and reduced dye was determined at a wavelength at which the enzyme shows no absorbance (>500 nm), and the amount of oxidized and reduced enzyme was determined at an isosbestic point for the dye.

Preparation of Apoprotein—The apoprotein of H69A BCO was prepared by overnight dialysis at 4 °C against 250 mM Tris buffer, pH 8.5, containing 2.5 M KBr, 20% glycerol, 0.3 mM EDTA, and 5 mM 2-mercaptoethanol. The sample was then desalted by gel permeation chromatography on a PD-10 column (Sephadex G-25) equilibrated with 100 mM potassium phosphate buffer, pH 7.5.

RESULTS

Spectral Properties and Reaction with Substrate—In comparison to wild-type BCO, the visible spectrum of the H69A mutant exhibits some differences both in the oxidized and reduced states (Fig. 1). In addition to the main band in the visible, oxidized H69A BCO has a shoulder at ≈395 nm and lacks the band in the near UV typical of wild-type BCO. The spectrum of the mutant also exhibits a substantial increase in the extinction coefficient of the band at 444 nm ($\epsilon = 16.07$ and 13.4 mM^{-1} cm^{-1} for H69A and wild-type BCO, respectively). Anaerobic addition of excess cholesterol results in full flavin reduction (Fig. 1), demonstrating that the noncovalently bound FAD mutant is competent in catalysis. The reduced form of H69A BCO is spectrally different from wild-type BCO in that the 376 nm band is replaced by a shoulder at ≈389 nm (Fig. 1). An anaerobic titration of H69A BCO with cholesterol is depicted in Fig. 1. The intercept of the initial slope of the titration curve with the maximal, observed absorbance change at 445 nm indicates a stoichiometry ≈1 for the reaction with substrate (16.3 nmol of enzyme and 18 nmol of substrate, *inset* of Fig. 1). Similar results were obtained with wild-type and (8Cl-FAD)-H69A BCO (not shown). During the anaerobic titration with H69A BCO, a small absorbance at ≈550 nm is evident that might be attributed to formation of a small quantity of anionic radical. Stabilization of the anionic semiquinone is typical for cholesterol oxidase (7) and for the family of flavoprotein oxidases (20). The amount of semiquinone (EFl_{seq}) formed by wild-type BCO upon anaerobic photoreduction is ≈70% of the extrapolated, possible maximum (Fig. 1). With the mutant this is reduced to ≤5% (Table I). Mutant and wild-type BCO display similar fluorescence emission spectra with maxima at 535 nm ($\lambda_{exc} = 450$ nm) and very low quantum yields (≈3% of the fluorescence of free FAD with wild-type BCO, and ≈10% with H69A BCO).

The pK_a value for the deprotonation of the flavin N(3)H position was estimated from the pH dependence of the visible spectrum of the oxidized enzymes. For wild-type BCO the pK_a is ≈11 (7), a value higher than that (pK_a ≈10) of free FAD (21). For H69A BCO a similar value was determined (pK_a ≈11.1 ±

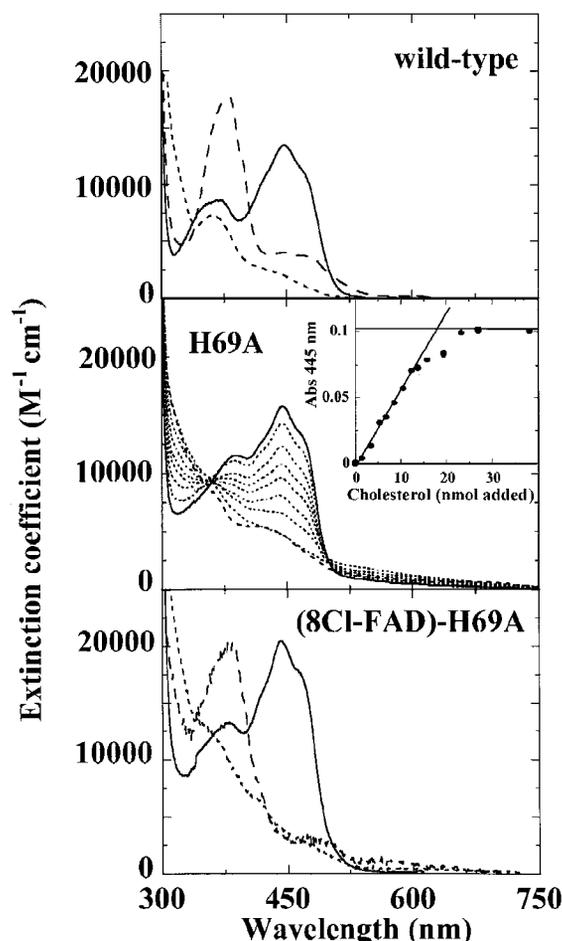*Covalent Cofactor Binding to Cholesterol Oxidase*

FIG. 1. **Spectral properties of wild-type, H69A, and (8Cl-FAD)-H69A mutant forms of BCO and anaerobic reaction of H69A BCO with cholesterol.** *Top panel: solid line,* oxidized wild-type BCO in 100 mM potassium phosphate buffer, pH 7.5, at 15 °C; *long dashed line,* semiquinone form generated by photoirradiation in the presence of 5 mM EDTA and 0.5 μM 5-deazaflavin; *dashed line,* fully reduced enzyme obtained from anaerobic reaction with 50 μM cholesterol. *Central panel*: *solid line,* oxidized H69A BCO. This sample (1.8 ml of solution of 9.05 μM H69A BCO, corresponding to 16.3 nmol of enzyme in 100 mM potassium phosphate buffer, pH 7.5) was anaerobically titrated with small additions of a cholesterol solution (0.375 mM in 50 mM potassium phosphate buffer, pH 7.5, containing 1% Thesit and 1% propan-2-ol), and at 15 °C. Selected spectra (*dotted line*) are shown for the following total concentrations of cholesterol (from top at 445 nm): 3.375, 5.25, 8.625, 10.5, 12.375, 19.5, and 38.25 nmol. *Inset,* dependence of the absorbance at 445 nm (upon correction for dilution) on the cholesterol concentration. The two linear parts of the saturation curve intercept at a ratio enzyme:substrate ≈1.1. *Dashed line*, fully reduced enzyme obtained from anaerobic reaction with 50 μM cholesterol. *Bottom panel*: —, oxidized (8Cl-FAD)-H69A BCO in 100 mM potassium phosphate buffer, pH 7.5, at 15 °C; *long dashed line*, semiquinone; and *dashed line*, fully reduced forms obtained by photoirradiation as described for wild-type BCO.

0.1). This confirms the absence of an effect of the covalent linkage specifically on this ionization process. The increase in pK observed for wild-type and H69A BCO compared with free FAD is likely to result from the microenvironment around the oxidized flavin pyrimidine: N(3)-H forms a tight H-bond (2.8 Å) with the backbone His[202] C(1)=O. The Arg[477] guanidinium group is at 3.1 Å from the flavin C(4)=O and can make hydrogen bond contacts directly with the side chains of both Glu[475] and Glu[311], this interaction likely neutralizing its charge.[2]

Stability—The stability of BCO in solution was studied by assessing the activity of each enzyme form at 25 °C in the presence of 50 mM potassium phosphate buffer, pH 7.5, and as a function of time. Both wild-type and H69A cholesterol oxi-

dases maintained more than 90% of the initial activity after 300 min at 25 °C (Fig. 2). The effect of organic solvents, surfactants, and ionic strength on CO catalysis is fundamental, since these factors affect the solubility of steroid substrates (very low in aqueous media), the micellar composition of the system and the enzyme activity (8, 10). Thus, we investigated the effect of propan-2-ol in the final concentration range 0–60% (v/v) on the time dependent activity of the two enzymes (shown in Fig. 2). Somewhat surprisingly, the (in)stability of the H69A mutant is quite comparable to that of wild-type BCO (Fig. 2), the main difference being the ≈30% residual activity observed with wild-type BCO at 20% propan-2-ol after 300 min, where H69A BCO is completely inactivated.

Kinetic Properties—The reaction of H69A BCO with cholesterol was studied using the H_2O_2 assay (7, 8, 10) and the EMTN method (15) under standard conditions (50 mM potassium phosphate buffer, pH 7.5, 1% Thesit, 1 or 10% propan-2-ol). With the first assay, and at a fixed (21%) O_2 concentration, a strong decrease in catalytic activity is evident for the H69A mutant: k_{cat} is reduced 30-fold and K_m increased 6-fold compared with the wild-type BCO (Table II). Although the k_{cat} values are quite similar at 1 and 10% (v/v) propan-2-ol, the increase in the alcohol concentration resulted in a sharp increase in the K_m value for cholesterol and with both wild-type and H69A COs. As wild-type BCO, the mutant catalyzes the isomerization reaction, *i.e.* the conversion of 5-cholesten-3-one, the assumed intermediate, into 4-cholesten-3-one, the final product (Scheme 1). Comparison of the rate of isomerization with those for oxidation of cholesterol (Table II) show that the Δ5–6 → Δ4–5 isomerization step is not rate-limiting for either enzyme, the k_{cat} for the isomerization step being 150 s^{-1} and 28 s^{-1} for wild-type and H69A BCO, respectively.

The kinetic mechanism of BCO with cholesterol as the substrate was recently studied by a combination of steady state and pre-steady state approaches (10). Based on these results, BCO was proposed to work via a ping-pong (binary complex) mechanism. The steady state kinetic behavior of H69A BCO was analogously studied using the EMTN method and cholesterol as the substrate. The oxidized enzyme was mixed aerobically with cholesterol and the change in flavin absorption monitored at 446 nm. A very rapid decrease in absorption was observed, amounting to ≈15% of the total change (data not shown). The value is significantly smaller than the one observed for the wild-type BCO (≈60%) which is due to the particular kinetic situation in which the rates of flavin reduction and reoxidation are similar (10). The smaller amplitude of the 446 nm absorbance change observed with the H69A BCO indicates that in the noncovalent mutant the rate of enzyme reduction is significantly decreased in comparison to the wild-type enzyme. The initial decrease in absorption was followed by a stationary phase, the duration of which depends on the initial cholesterol concentration and which leads to the fully reduced enzyme as the final state. The 446-nm traces were analyzed as a function of oxygen concentration according to Ref. 15; the kinetic parameters obtained are given in Table III. The double-reciprocal (Lineweaver-Burk) plot of the turnover number as a function of substrate concentration always gave a set of parallel lines, suggesting that a ping-pong mechanism is still operative for the H69A BCO. EMTN experiments confirm that the absence of covalent flavin linkage in BCO results in a large decrease in the turnover number and in a large increase in the Φ_{Chol} term of the steady state equation (Table III).

Preparation of H69A BCO Apoprotein and Reconstitution with FAD—Several methods were explored in order to obtain the apoenzyme of H69A BCO. We established that mild dialysis in the presence of 2.5 M KBr and 20% glycerol produces good

TABLE I
Spectral and redox properties of wild-type, H69A, and
(8Cl-FAD)-H69A forms of BCO

The enzyme solutions used for the absorbance spectra were ~10 μM in 100 mM potassium phosphate buffer, pH 7.5, at 25 °C; those for fluorescence spectra were ~0.15 μM in the same buffer. Reduced enzymes (same concentration) were obtained under anaerobic conditions after the addition of 50 μM cholesterol in 100 mM potassium phosphate buffer, pH 7.5, 1% Thesit, and 1% (v/v) propan-2-ol. The semiquinone forms (same enzyme concentration) were achieved by anaerobic photoreduction in the presence of 5 mM EDTA and 0.5 μM 5-deazaflavin. The redox potentials were determined spectroscopically, using the xanthine and xanthine oxidase system proposed by Massey (19). The measurements were made in 100 mM potassium phosphate buffer, pH 7.5, at 15 °C in the presence of cresyl violet ($E_m = -176$ mV) and phenosafranine ($E_m = -239$ mV).

	Wild-type[a]	H69A	(8Cl-FAD)-H69A
Spectral properties			
EFl$_{ox}$			
λ_{max} (nm)	275, 368, 448	274, 389, 444	272, 380, 444
ε (mM^{-1} cm^{-1})	170, 8.7, 13.4	161.3, 11.3, 16.07	403.6, 13.25, 20.5
Absorbance ratios	12.7, 0.65, 1	10.1, 0.7, 1	19.7, 0.65, 1
Fluorescence emission λ_{max} (nm)[b]	325, 525	336, 526	334, 527
EFl$_{red}$			
λ_{max} (nm)	268, 362	271, 406	369, 438
ε (mM^{-1} cm^{-1})	174.4, 7.3	170.6, 5.5	12.28, 6.88
EFl$_{seq}$			
λ_{max} (nm)	382, 445	BD[c]	384, 444
ε (mM^{-1} cm^{-1})	17.7, 4.0	BD	23.47, 11.18
Semiquinone measured (%)	70	<5	≈40
Redox properties			
E_m (mV)	−101	−204	−160

[a] Data from Ref. 7.

[b] λ excitation is 285 and 450 nm, respectively.
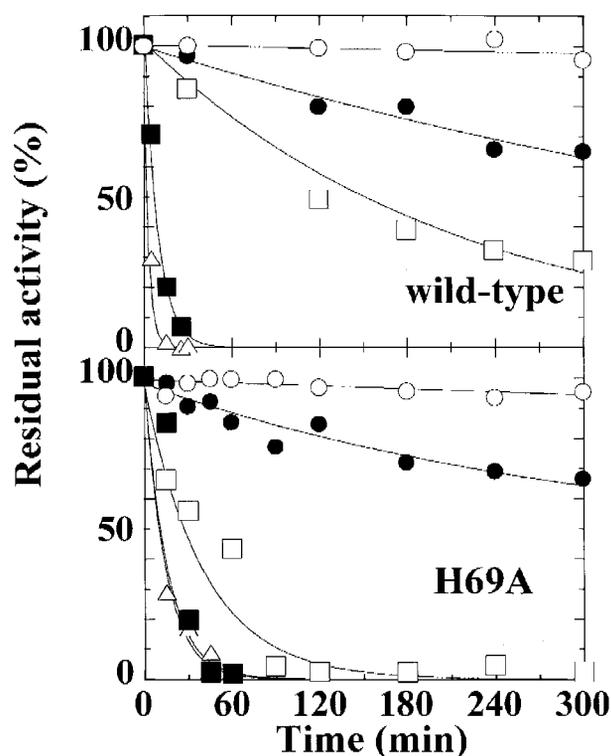
[c] BD, below detection.

FIG. 2. **Stability of wild-type and H69A BCO and effect of propan-2-ol.** The enzymes, 0.1 mg protein/ml, in 50 mM potassium phosphate buffer, pH 7.5, and at 25 °C were incubated at the following concentrations of propan-2-ol: 0% (○); 10% (●); 20% (□); 30% (■); and 40% (△) (all final concentrations).

yields and a preparation that could be reactivated with a >80% recovery (the monovalent bromide anion competes with coenzyme, weakening the binding of flavin to apoproteins, whereas glycerol favors protein stabilization). The properties of the reconstituted holoenzyme are nearly identical to those of the starting H69A BCO with respect to spectral properties and specific activity (~0.8 units/mg of protein). Binding of FAD to the apoprotein was measured by following the quenching of protein fluorescence (emission at 340 nm), as well as by following the increase in flavin fluorescence (emission at 530 nm) during the titration of 0.3 μM apoprotein with FAD. A K_d value of 3.7×10^{-8} M was estimated, indicating a rather tight binding. It is worth noting that the data are consistent with a 1:1 molar ratio of apoprotein:FAD binding. The kinetics of reconstitution of the H69A apoprotein-FAD complex were studied under pseudo-first order conditions (10–150-fold excess of FAD or apoprotein) by following the time course of quenching of protein or flavin fluorescence. In both cases the reaction curve is biphasic and is best fit by two exponentials. Fig. 3 shows the time course of the total change in protein fluorescence after mixing H69A apoprotein with excess of FAD: both the initial fast phase and the slow secondary change proceed according to a first-order exponential processes. Interestingly, at the end of the reaction, FAD fluorescence was quenched to 10–15% of the value of free FAD (see above). The observed first-order rate constant of the fast phase (k_{obs1}) depends linearly on the concentration of the FAD (*inset* of Fig. 3). The process can thus be described by two subsequent, irreversible steps with constants $k_1 = 1.5 \times 10^4$ M^{-1} s^{-1}, and $k_2 = 2 \times 10^{-4}$ s^{-1}. Although in this context the reversal rate constants can be assumed to be close to zero, they have a finite value since the holoenzyme reconstitution process is reversible. The observed first-order rate constant for the second (slow) phase was not dependent on the apoprotein concentration and represents a sequential rearrangement to yield the holoenzyme form. The protein fluorescence change associated with the second phase corresponds to ≈25% of the total quenching during the reconstitution of H69A apoprotein with FAD. Very similar results were obtained by monitoring changes in protein fluorescence after mixing apoprotein with a large excess of FAD (10–150-fold).

Formation of active H69A holoenzyme during the incubation of apoprotein with excess FAD at 15 °C under the experimental conditions used for the fluorometric analysis was followed using the *o*-dianisidine activity assay. The increase in absorbance at 440 nm shows only a short lag, activity being fully regained during the first phase as defined by the fluorescence decrease experiments. The nature of the second rearrangement is unknown.

TABLE II
Kinetic parameters for the reaction of wild-type, H69A, and (8Cl-FAD)-H69A forms of BCO with cholesterol (oxidation)
and 5-cholesten-3-one (isomerization)

Measurements were in 50 mM potassium phosphate buffer, pH 7.5, at 25 °C. Assay methods: A, rate of H_2O_2 formation with *o*-dianisidine and horseradish peroxidase; B, spectrophotometric detection of 4-cholesten-3-one formation at 240 nm.

	Conditions	Oxidation (A)		Isomerization (B)	
		k_{cat}	$K_{m,chol}$	k_{cat}	$K_{m,5\text{-chol-3-one}}$
		s^{-1}	mM	s^{-1}	mM
Wild-type[a]	1% Thesit, 1% propan-2-ol	48	0.04		
	1% Thesit, 10% propan-2-ol	43	0.25	150	0.37
H69A	1% Thesit, 1% propan-2-ol	1.4	0.26		
	1% Thesit, 10% propan-2-ol	1.4	1.3	28	0.35
(8Cl-FAD)-H69A	1% Thesit, 1% propan-2-ol	6.7	0.95		
	1% Thesit, 10% propan-2-ol	5.2	1.6	20	0.58

[a] Data from Ref. 7.
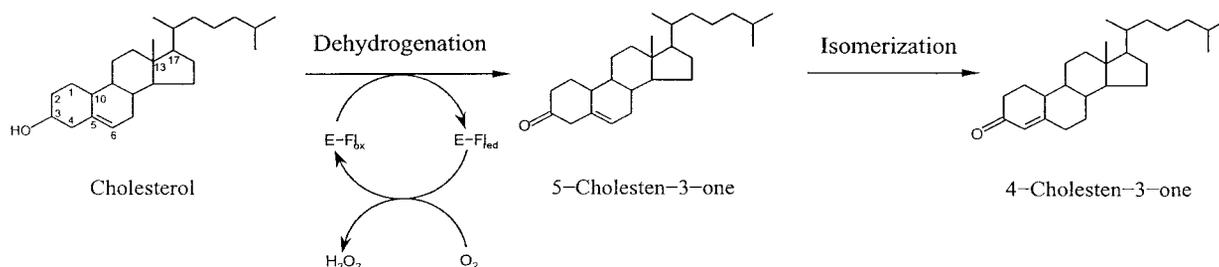
SCHEME 1. **Reactions catalyzed by BCO.** In the first step (*left-hand side*) cholesterol is dehydrogenated to the intermediate 5-cholesten-3-one; the redox equivalents are used to reduce the oxidized flavin, and are subsequently transferred onto oxygen (oxidase reaction). Subsequently a ($\Delta5-6 \rightarrow \Delta4-5$) isomerization of the intermediate to form 4-cholesten-3-one occurs (*right-hand side*) at the level of oxidized enzyme flavin. Note that with BCO the isomerization reaction and reoxidation of reduced enzyme by oxygen are substantially faster than the dehydrogenation, the latter being rate-limiting.

TABLE III
Comparison of steady-state coefficients obtained for the reaction of wild-type and H69A BCO with cholesterol as
substrate in stopped-flow experiments

Measurements were in 50 mM potassium phosphate buffer, pH 7.5, 1% propan-2-ol, and 1% Thesit, at 25 °C.

	Lineweaver-Burk pattern	k_{cat}	Φ_{Chol}	$K_{m,Chol}$	Φ_{O_2}	K_{mO_2}
		s^{-1}	$M\,s \times 10^{-6}$	mM	$M\,s \times 10^{-6}$	mM
Wild-type[a]	Parallel	19.2	12	0.21	7.3	0.14
H69A	Parallel	0.55	495	0.47	25	0.45

[a] Data from Ref. 10.

Redox Potentials—A preliminary indication of a modification of the redox properties of H69A BCO arises from the sulfite reactivity. Addition of sulfite to oxidized wild-type BCO results in essentially complete bleaching of the oxidized flavin spectrum (7). Under the same conditions, the reaction of the H69A mutant is very slow and is not completed after 41 h, reflecting a $K_d \approx 115$ mM that compares to 0.14 mM for wild-type BCO. These properties are comparable to those observed with CO from *S. hygroscopicus* that contains noncovalently linked FAD (7).

Therefore, the redox potential of the H69A BCO was measured in order to assess the effect of the mutation on the thermodynamic properties of the flavin. When the xanthine oxidase-mediated reduction of H69A BCO was monitored in the absence of a reference dye, the percentage of semiquinone formed during the reduction (Fig. 4) was close to zero (~70% with wild-type BCO), *i.e.* the values of the potentials for transfer of each single electron were similar. The estimated separation between the potentials is ≤15 mV (22) and, thus, the redox potentials for each single electron transfer cannot be determined accurately. The midpoint potential of the oxidized/reduced forms ($E_m = (E_1 + E_2)/2$) was determined by using phenosafranine and cresyl violet as reference dyes. The redox potential difference with respect to the dye was calculated by plotting $\log([dye_{ox}]/[dye_{red}])$ as a function of $\log([E_{ox}]/[E_{red}])$ (18): this plot has a slope ≈0.88 (data not shown) which is close to the theoretical value of 1.0 for a two-electron reduction process. H69A BCO has a midpoint redox potential, E_m, which is about 100 mV more negative than wild-type BCO (Table I). This change should influence catalysis, since the midpoint potential of −200 mV makes enzyme reduction by cholesterol, the rate-limiting step with wild-type BCO (10), thermodynamically unfavorable. A decrease in the reoxidation rate is not an issue because the O_2/H_2O_2 couple is more positive (+300 mV at pH 7) than the $FAD/FADH_2$ couple. The results indicate that the lack of the flavin covalent link results in a ≈100 mV shift of the midpoint redox potential (−101 mV *versus* −204 mV for the wild-type and H69A, respectively). A similar change in redox potentials has been recently reported for vanillyl-alcohol oxidase (23).

Properties of (8Cl-FAD)-H69A BCO—8-Chloro-FAD was chosen to reconstitute H69A apoprotein because of its higher potential with respect to unmodified FAD ($E_m = -152$ mV *versus* −207 mV). Binding of the analogue was followed by the quenching of the protein fluorescence and is similar ($K_d = 1.6 \times 10^{-8}$ M) to that of normal FAD. The spectra of the
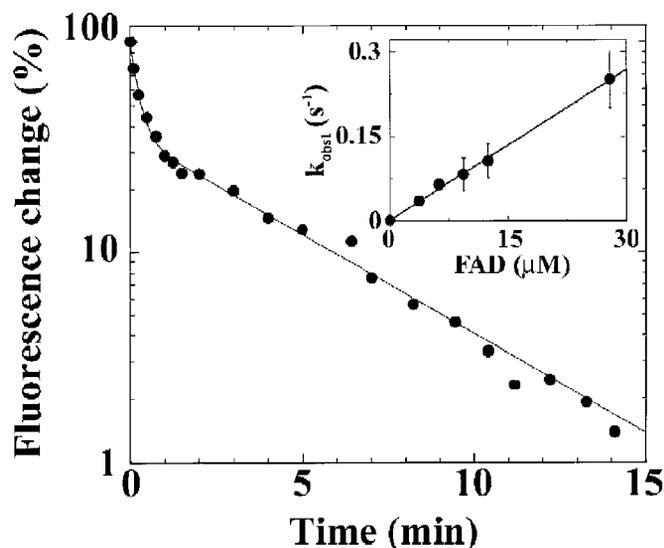
FIG. 3. **Time course of fluorescence quenching during the reconstitution of H69A BCO apoprotein with FAD.** Protein fluorescence change (arbitrary units) after the mixing of 1.25 μM apoprotein solution with 12.6 μM FAD solution (final concentrations) in 100 mM potassium phosphate buffer, pH 7.5, at 15 °C. Excitation wavelength: 285 nm; emission wavelength: 340 nm. *Inset,* effect of FAD concentration on the observed first-order rate constant (k_{obs1}) of the reconstitution of 0.63 μM H69A BCO apoprotein. The values of k_{obs1} were calculated from the slope of the first rapid phase of the logarithmic plots as shown in the *main graph*, after subtraction of the second, slow phase.
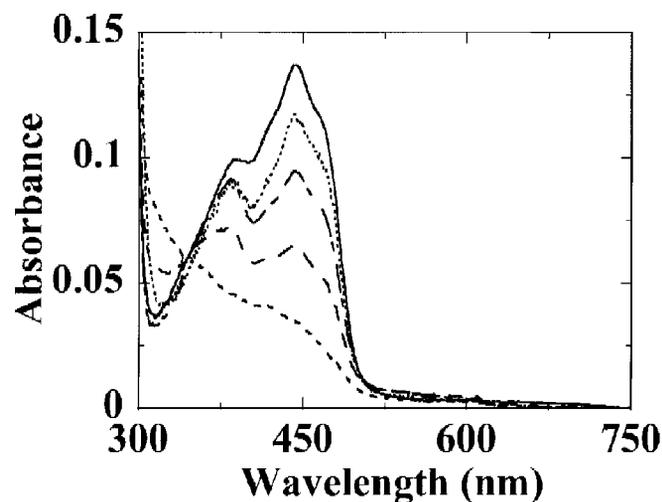

FIG. 4. **Time course of anaerobic reduction of H69A BCO using the xanthine/xanthine oxidase system.** Selected spectra obtained during the course of the anaerobic reduction of 8.7 μM H69A BCO in 100 mM potassium phosphate buffer, pH 7.5, in the presence of 200 μM xanthine and 5 μM BV, at 15 °C: *solid line,* before addition xanthine oxidase; *dotted line,* 12.5 min, *dot dash line,* 22.5 min; *long dashed line,* 42.5 min, and *dashed line,* 62.5 min after addition of 10 nM xanthine oxidase.

oxidized and cholesterol-reduced (8Cl-FAD)-H69A BCO are intermediate in shape compared with those of wild-type and H69A BCO (Fig. 1). This suggests that the peculiar spectral properties of H69A BCO result from a combination of electronic and structural effects. The spectrum of the radical intermediate, on the other hand, is similar to that of wild-type BCO (Fig. 1), suggesting that electronic factors are decisive for the properties of the semiquinone. With (8Cl-FAD)-H69A BCO, a higher amount of flavin semiquinone (~40%) is observed than is formed with H69A BCO. Sulfite reactivity strongly differen-

tiates (8Cl-FAD)-H69A BCO from that containing normal FAD. The former reacts rapidly to form the flavin N(5)-covalent adduct with a $K_d \approx 3.6$ mM that is intermediate between the binding constants for wild-type and H69A BCO containing unmodified FAD. Catalytic activity of the (8Cl-FAD)-H69A BCO is 3.5-fold higher than that observed with normal FAD reconstituted mutant, while the rate of isomerization is unaltered by the flavin substitution (Table II).

The redox potential of (8Cl-FAD)-H69A BCO is −160 mV and was determined using the xanthine/xanthine oxidase reducing system and cresyl violet as mediator. This potential is thus ≈45 mV more positive than that of H69A containing normal FAD. Hence, the higher catalytic activity of the (8Cl-FAD)-H69A BCO is due largely to its more positive redox potential.

DISCUSSION

The present results demonstrate that covalent flavinylation is not a prerequisite for CO protein folding and for efficient binding of FAD. The latter could be removed reversibly as previously reported for other flavoproteins (24). In the binding experiments a $K_d \approx 2 \times 10^{-8}$ M for both FAD- and (8Cl-FAD)-apoprotein complexes was determined. The lack of the covalent flavinylation due to the substitution of H69A does not abolish the tight binding of the coenzyme to BCO. This observation demonstrates that a functional holoenzyme can be obtained from the noncovalent interactions of the coenzyme with the apoprotein moiety. Binding of FAD to H69A apoprotein appears to be a two-stage process, in which only the first (rapid) phase depends on the FAD or apoprotein concentration, a reconstitution process similar to that of many other noncovalent flavoproteins (24, 25). The rapid initial phase observed in the fluorimetric experiments leads directly to the fully functional enzyme since it has the same rate as the recovery of CO activity. The second step might reflect secondary conformational changes of the holoenzyme (24).

Our results are compatible with the concept that covalent flavinylation as such is not a requirement for catalysis. The observed effects can be attributed largely to the decrease in the redox potential.

From a kinetic point of view based on EMTN measurements, wild-type and H69A BCO function by a binary complex mechanism which is similar to that reported previously for intact enzyme (10), the main difference being a ≈30-fold decrease in the rate of flavin reduction. By comparison of the parameters of cholesterol dehydrogenation with those for the isomerization of 5-cholesten-3-one (Table II, see also Scheme 1), it is apparent that the latter is never rate-limiting.

Interestingly, the E_m value for H69A BCO (−204 mV) is very close to that of CO from *S. hygroscopicus* (−217 mV) that contains noncovalent FAD (7) and to that of free FAD (−207 mV). The effect of the 8α-(N3)histidyl substitution on the redox potential of free flavins consists of an increase of ≈30 mV (26). It should be noted, however, that this effect is strongly pH dependent and reflects the ionization state of the histidine substituent (27). These authors have pointed out that a role of histidine substitution might indeed be the modulation of the redox potential of the flavin-bound enzyme. This suggestion appears to be borne out by the present results. On the other hand the rates of flavin reduction for BCO and SCO (235 s^{-1} and 232 s^{-1} at 1% Thesit and 10% propan-2-ol, respectively) (10) do not correlate with the corresponding midpoint redox potentials. This apparent inconsistency might be due to the mentioned potentials having been determined for the free enzyme forms as opposed to the enzyme-substrate systems. Furthermore, it is to be expected that additional factors such as the nature and position of functional groups involved in catalysis

and parameters derived from the overall structures are important in determining the absolute values of catalytic steps.

Effects similar to those described here have been reported recently for vanillyl alcohol oxidase, where an analogous H422A mutation and the consequent absence of covalent flavin linkage lead to a ~120 mV more negative E_m potential (23). Importantly, with vanillyl alcohol oxidase the three-dimensional structural data suggest that the effect on the midpoint redox potential is not due to structural alterations and that the flavin covalent link does not induce specific structural features. We anticipate a similar verification from the results of the study of the crystal structure of BCO and H69A BCO. The main difference between BCO and vanillyl oxidase is, thus, the great stabilization of the semiquinone form in vanillyl alcohol oxidase (23), whereas the two-electron transfer is thermodynamically favored with H69A BCO. The thermodynamic effects induced by the covalent flavin linkage can be mimicked successfully using 8Cl-FAD, an analogue with a 65-mV higher potential than that of normal FAD. This is also manifested in the sulfite reactivity. The holoenzyme of H69A BCO reconstituted with the "high potential" 8-chloro-FAD analogue shows a ≈4-fold higher catalytic activity than the corresponding FAD derivative (Table II) which probably stems from the ≈45 mV more positive redox potential (Table I). Thus, within the same enzyme system a correlation of rate of reduction with the redox potential appears to be verified. On the other hand, the rate of $\Delta 5–6 \rightarrow \Delta 4–5$ isomerization that does not involve redox processes is unaffected by the coenzyme replacement (Table II).

In conclusion, it appears that the flavin 8α-substitution with a (N3)histidine in BCO is an important factor in the modulation of its redox properties as a way to increase its oxidation power. Why there are two CO enzymes in the genus *Brevibacterium* that are differentiated by the presence of the covalent flavin linkage is not known. Whether this is a fortuitous event or reflects evolutive pressure (*i.e.* due to the different environments in which the two COs reveal their catalytic function) is an interesting aspect awaiting elucidation.

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