Dissecting the Structural Determinants of the Stability of Cholesterol Oxidase Containing Covalently Bound Flavin*

Cholesterol oxidase from Brevibacterium sterolicum is a monomeric flavoenzyme catalyzing the oxidation and isomerization of cholesterol to cholest-4-en-3-one. This protein is a class II cholesterol oxidases, with the FAD cofactor covalently linked to the enzyme through the His\(^{69}\) residue. In this work, unfolding of wild-type cholesterol oxidase was compared with that of a H69A mutant, which does not covalently bind the flavin cofactor. The two protein forms do not show significant differences in their overall topology, but the urea-induced unfolding of the H86A mutant occurred at significant lower urea concentrations than wild-type (−3 versus −5 \(\text{M}\), respectively), and the mutant protein had a melting temperature 10–15 °C lower than wild-type in thermal denaturation experiments. The different sensitivity of the various spectroscopic features used to monitor protein unfolding indicated that in both proteins a two-step (three-state) process occurs. The presence of an intermediate was more evident for the H69A mutant at 2 \(\text{M}\) urea, where catalytic activity and tertiary structure were lost, and new hydrophobic patches were exposed on the protein surface, resulting in protein aggregation. Comparative analysis of the changes occurring upon urea and thermal treatment of the wild-type and H69A protein showed a good correlation between protein instability and the elimination of the covalent link between the flavin and the protein. This covalent bond represents a structural device to modify the flavin redox potentials and stabilize the tertiary structure of cholesterol oxidase, thus pointing to a specific meaning of the flavin binding mode in enzymes that carry out the same reaction in pathogenic versus non-pathogenic bacteria.

Bacterial cholesterol oxidase (CO, EC 1.1.3.6) is a monomeric bifunctional FAD-containing flavoenzyme that catalyzes the oxidation of \(3\beta\)-hydroxysteroids and the isomerization of the intermediate, \(\Delta^{5-6}\)-ene-3\(\beta\)-ketosteroid (cholest-5-en-3-one) to produce \(\Delta^{3-4}\)-ene-3\(\beta\)-ketosteroid (cholest-4-en-3-one). Bacteria that produce CO can be classified into two classes: non-pathogenic (e.g. Streptomyces and fast-growing Mycobacteria; these bacteria utilize cholesterol as their carbon source) and pathogenic bacteria (e.g. Rhodococcus equi and slow-growing Mycobacteria). Pathogenic bacteria require CO for infection of the host macrophage probably because of its ability to alter the physical structure of the membrane by converting cholesterol into cholest-4-en-3-one.

The crystal structure of COs belonging to two distinct structural classes (1) have been solved by the Vrielink laboratory (2–4). Members classified as type I, such as those from Streptomyces sp. SA-COO and R. equi (5) have their cofactor tightly but non-covalently bound to the enzyme. A second CO from Brevibacterium sterolicum classified as type II, is completely different. In this enzyme the cofactor is covalently linked to the protein via a bond between the 8-methyl group of the isoalloxazine ring and the ND1 atom of His\(^{69}\).

Although COs of class I and II share the same catalytic activity, they show no sequence homology, differ in the flavin binding mode, and have significant differences in their redox and kinetic properties (6, 7). Many proteins use tightly bound cofactors to perform their biological functions, and up to now some 30 flavoenzymes have been reported to contain flavin covalently linked to a histidine, cysteine, or tyrosine side chain (8, 9). The redox properties of the flavin are modulated through a specific protein microenvironment, to suit the particular requirements of the redox reaction. Previous studies on the C406A mutant of monoamine oxidase suggested that covalent flavin binding markedly improves the structural stability of the enzyme (9, 10). The covalent CO we studied here belongs to the family of vanillyl-alcohol oxidase (11), which contains a fold proposed to favor covalent flavinylation.

To elucidate the function of covalent flavin linkage in this CO, we studied a mutant in which the His\(^{69}\) → Ala exchange prevents formation of the histidyl-FAD bond (the numbering refers to the mature protein, and this residue corresponds to His\(^{121}\) in the full-length enzyme) (12). The mutant retains catalytic activity and carries out also the isomerization step of the intermediate cholest-5-en-3-one to the final product, but the non-covalent flavin binding results in a 35-fold decrease in turnover rate and in a much lower flavin midpoint redox potential (~204 mV, compared with ~101 mV for wild-type). We concluded that the flavin 8α-linkage to a N1 histidine is a pivotal factor in the modulation of the redox properties of this CO to increase its oxidative power (12).

In view of the biotechnological applications, there is considerable interest in this flavoenzyme. CO is widely used for determining serum cholesterol levels in the diagnosis of atherosclerosis and other lipid disorders (13). Also, CO is a potent larvicidal and has been developed as an insecticide against Coeloptera (14). Furthermore, CO homologues are secreted by life-threatening pathogens (R. equi, Mycobacterium tuberculosis, Mycobacterium leprae) that have been proposed to play a
role in the lysis of macrophages and leukocytes leading to the characteristic lesions found in infected humans and animals (15). In particular, R. equi is an opportunistic pathogen in humans, causing tuberculosis-like lung infections in severely immunocompromised patients, such as human immunodeficiency virus-infected individuals. As these enzymes are unique to bacteria, they represent a potential target for a new class of antibiotics.

In the framework of a project devoted to the analysis of flavoenzyme structure and function, we are using CO from B. sterolicum as a model for protein folding and coenzyme binding studies. The DNA coding for the protein has been synthesized and can be efficiently expressed in Escherichia coli, the structure of the wild-type enzyme is known (4) and that of the H69A mutant has been recently completed. In this paper we dissect the contribution of the covalent link of the flavin cofactor on the stability and folding process of CO.

EXPERIMENTAL PROCEDURES

Materials and Enzymes—Cholesterol and Thesit® (dodecyl polyethylene glycol succinate), n = 9–10) were purchased from Roche Diagnostics. All other reagents were of the highest commercially available purity. Wild-type and H69A recombinant COs were obtained from Roche Diagnostics. Enzyme concentration was determined using the known extinction coefficients (16.1 and 13.4 mm⁻¹ cm⁻¹ for H69A and wild-type CO, respectively, at 455 nm (12).

Enzymatic Activity—Cholesterol oxidase activity was assayed monitoring H₂O₂ production at 440 nm (ε₄₄₀ = 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 (6, 7). Enzyme activity was assayed in 50 mM potassium phosphate buffer, pH 7.5, containing 1% (v/v) propan-2-ol, at 25 °C. The activity was determined using the same spectrophotometric assay on protein samples previously incubated at 15 °C for 60–90 min in the presence of various denaturant concentrations.

Absorption and Fluorescence Measurements—UV visible absorption spectra were recorded with a Jasco V-560 spectrophotometer in 100 mM potassium phosphate buffer, pH 7.5, at 25 °C. Flavin and protein fluorescence measurements at fixed temperatures were performed in a Jasco FP-750 instrument equipped with a thermostatted cell holder using a 1-ml cell. Trypsinophan emission spectra were taken from 300 to 400 nm using excitation wavelengths of 250 or 298 nm. Flavin emission spectra were recorded from 475 to 600 nm using an excitation wavelength of 450 nm; 10-nm band widths were used for excitation and emission, respectively. Steady-state fluorescence measurements were performed at 15 °C in 100 mM potassium phosphate buffer, pH 7.5, at 0.1 mg/ml protein concentration. All spectra were corrected by subtracting the emission from the buffer. Temperature-ramp experiments were performed using a software-driven, Peltier-based temperature controller, which allowed reproduction at the same temperature gradient (0.5 °C/min) used in circular dichroism and calorimetric studies. Fixed wavelength measurements were taken at 340 and 526 nm for tryptophan and flavin fluorescence, respectively. Circular dichroism (CD) spectra were recorded on a J-810 Jasco spectropolarimeter, also equipped for the emission of the buffer. Temperature-ramp experiments were performed in an automated protein sequencer (Procise 492, Applied Biosystem).

Calorimetry—Calorimetric measurements were carried out on 23 μM (1.5 mg/ml) protein in 100 mM potassium phosphate, pH 7.5, or in 100 mM Tris-HCl, pH 8.5. A third generation Setaram Micro-DSC apparatus was used, which is suitable for diluted solutions of biological macromolecules in the temperature range from −20 to 120 °C. Scan rates of 0.5 and 0.1 °C/min were used. Data were analyzed by means of the THESEUS software (16). The excess molar heat capacity Cₚₑₓ(T) and Cₚₑₓ(T) or Cₚₑₓ(T), i.e. the difference between the apparent molar heat capacity Cₛ(T) of the sample and the molar heat capacity of the "native state," Cₛ(∞)(T), was recorded across the scanned temperature range. Cₛ(∞)(T) was obtained according to published procedures (17) by linear regression of experimental Cₛ(T) data in the pre-denaturation region (namely, for T < Tₛ, where Tₛ is the highest temperature at which only the native protein is present). The overall calorimetric denaturation enthalpy ΔH was determined by integration of Cₛ(T) across the denaturation (Tₛ + Tᵢ) range, where Tᵢ is the final temperature of the denaturation process, taking into account that the baseline has a sigmoidal trend across the same temperature range. Such a trend can be obtained by assigning a weight factor equal to the ratio incremental area/total area, drawn from the above integration, and iterating the overall process until convergence (16). As a first step of the iteration procedure, a straight line passing through Cₛ(Tᵢ) and Cₛ(Tₛ) was used as the tentative baseline. All the thermograms reported in this paper were accordingly scaled. Because of the presence of aggregation phenomena, the heat capacity drop from ΔCₛ(Tᵢ) which corresponds to the Cₛ change at passing from native to the denatured state across the signal was affected by a rather large error and was therefore not taken into account. The procedures described above leveled off any uncertainty about ΔCₛ(Tᵢ) and allowed a reliable evaluation of Cₚₑₓ(T) and of the corresponding enthalpy. The theoretical models used to fit the experimental data were tested through the non-linear Levenberg-Marquardt method (18).

ANS Binding—Wild-type and ANS Binding and ANS Binding Experiments—The unfolding equilibrium of CO was determined by following the changes in flavin and protein fluorescence, as well as in near- and far-UV CD signals, as detailed above. To establish the time required to reach equilibrium, the fluorescence intensity was measured as a function of time until no further changes are observed (60–90 min at 15 °C). Each point in urea denaturation curves was determined on individual samples, prepared by mixing appropriate volumes of a concentrated protein stock, 8 M urea in buffer, and 100 mM potassium phosphate, pH 7.5 or 8.5.

ANS binding experiments were carried out at 15 °C and at 0.1 mg/ml protein concentration. For unfolding experiments, protein samples were incubated for 60 min at 15 °C in buffer containing different concentrations of urea, and then folded by 10-fold dilution in 100 mM potassium phosphate, pH 7.5, at 15 °C. The refolding yield was measured by monitoring protein and flavin fluorescence (using the values for the native and fully denatured enzymes as reference), and by the recovery of enzymatic activity with respect to the untreated protein (12).

Data Analysis—In all cases, the unfolding curves were analyzed using a two-state mechanism. At first, unfolding curves for the N → U transition were normalized to the apparent fraction of the unfolded form, Fₒ, using the following equation (19),

\[ Fₒ = (Y - Yₒ)(Yₒ - Y) \tag{1} \]

Y is the observed variable parameter, and Yₒ and Y are the corresponding values for the native and fully unfolded conformations, respectively. The difference in free energy between the folded and the unfolded state, ΔG, was calculated by following the equation,

\[ ΔG = -RT \ln K = -RT \ln \left[ \frac{Fₒ}{(1 - Fₒ)} \right] \tag{2} \]

K is the equilibrium constant, R is the gas constant, and T is the absolute temperature. The data were analyzed assuming the free en-

1 L. Pollegioni, unpublished results.
2 A. Vriezen, personal communication.
3 The abbreviation used is: ANS, 8-anilinonaphthalene-1-sulfonate.
Unfolding of Cholesterol Oxidase

Recombinant wild-type and H69A COs were purified as holoenzymes showing significant differences in their visible absorbance spectra because of the different binding mode of the flavin cofactor (12). In particular, in addition to the main band at ~445 nm (extinction coefficients of 16.1 and 13.4 mM⁻¹ cm⁻¹ for H69A and wild-type CO, respectively), the mutant CO shows a shoulder at ~395 nm and lacks the band in the near-UV typical of the wild-type enzyme.

Some other spectral properties related to protein folding also distinguish the two CO forms. Tryptophan fluorescence (following excitation at 280 nm) shows an emission maximum at 331 and 335 nm for wild-type and H69A CO, respectively, with a higher intensity for H69A than for wild-type CO, indicating a different relevance of quenching interactions between tryptophans and nearby side chains in the two proteins. The emission fluorescence of the FAD cofactor (following excitation at 450 nm) is also different for the two CO forms. The emission maximum is observed at 512 and 524 nm for wild-type and H69A CO, respectively, and the intensity of flavin fluorescence is 7-fold higher for the mutant protein. The lower fluorescence of the wild-type protein at pH 7.5 with respect to the H69A mutant may ensue from deprotonation of the 8a-histidylflavin, which results in a marked diminution in observed flavin fluorescence (21, 22) and occurs in the pKₐ range 5.4–6.2, depending on the source of CO used (22).

For both proteins, unfolding by urea is accompanied by an increase in both flavin and tryptophan fluorescence. Only tryptophan fluorescence had similar values in the two fully unfolded proteins (see below). Of course, unfolded wild-type CO does not release the flavin cofactor, which is covalently linked to the protein, whereas flavin fluorescence (following excitation at 450 nm) is indeed for the fully unfolded H69A mutant. Flavin fluorescence is indeed ~12-fold lower in unfolded wild-type CO with respect to that of the fully unfolded mutant.

Far-UV CD spectra of wild-type and H69A CO did not reveal any major difference in the features related to the secondary structure of the two proteins, whereas some minor differences are evident in the near-UV CD spectra (e.g. higher ellipticity at 285 nm for the H69A mutant and at 253 nm for the wild-type CO, respectively) pointing to a slight alteration of the tertiary structure in the mutant protein, as also indicated by the protein fluorescence data discussed above. Following the addition of ~7 M urea, the signals observed in the far- and near-UV CD spectra of both wild-type and H69A CO were abolished.

Equilibrium Unfolding Studies

The stability of wild-type and H69A CO toward chaotropes was studied by equilibrium unfolding measurements by following different spectroscopic signals and the catalytic activity after equilibration in the presence of increasing urea concentration.

Protein Fluorescence—The intrinsic fluorescence of tryptophan residues was used as probe of the protein unfolding.

Because in CO there are 19 tryptophans, the overall changes in fluorescence are indicative of global changes in protein structure. At increasing urea concentrations, the two CO forms showed a different increase in the intensity of the protein emission that was anyway complete at 7 M urea (Fig. 1A). Addition of urea also caused the emission maximum of tryptophan fluorescence to shift from 331 nm for wild-type and 335 nm for H69A CO to 354 nm at 7–8 M urea, a change that for both enzyme forms anticipated the change in fluorescence intensity (Fig. 1A). The observed fluorescence red shift is considered a marker of the transfer of tryptophan side chains to a more polar environment upon protein unfolding (23).

Plots of changes in the intensity of protein fluorescence at equilibrium as a function of the urea concentration apparently suggest a simple two-state transition (Fig. 1A). The free energy of unfolding, ΔG, was calculated according to Equation 2. The free energy of unfolding in the absence of the denaturant (ΔGₒ) can be obtained by extrapolation of ΔG to 0 denaturant concentration using Equation 3 (see Table I). For both COs, changes in the wavelength of emission maximum at increasing urea concentrations were different from those of protein fluorescence intensity, and occurred at lower urea concentrations (Cₒ for wild-type and H69A CO, respectively). This indicates that the overall urea-induced denaturation of CO might be a more complex process, pointing to the presence of possible unfolding intermediates.

Flavin Fluorescence—Increasing urea concentrations had a
different effects on flavin fluorescence in the two proteins. Flavin fluorescence of the H69A mutant at increasing urea concentrations paralleled the increase observed for protein fluorescence (see Fig. 1, A and B, and Table I), and attained values similar to that of the free flavin at ~7 M urea. All together, both protein and flavin fluorescence are indicative of a two-state process for both proteins, and do not provide direct evidence for an unfolding intermediate.

The effect of increasing urea concentrations on the protein and flavin fluorescence signals of wild-type and H69A CO was also investigated at pH 8.5, where the native enzymes retain their catalytic activity in full. As reported in Table I, the increase in pH resulted in a shift of the unfolding curve toward higher Cm values (relative increase, 0.6–1.1 M) for wild-type CO, whereas unfolding of the H69A mutant was affected to a much more limited extent (relative increase, 0.1–0.3 M).

**CD Spectra**—When urea-induced loss of secondary structural elements was monitored by following the changes in ellipticity at 220 nm (Fig. 2A), the CD signal of wild-type and H69A CO showed a different sensitivity to the chaotrope. Loss of the 220-nm signal started at a concentration of urea >1.5 M for the mutant protein, but only at >4.0 M urea for wild-type.

Addition of urea also has a different effect on the tertiary structure of wild-type and H69A CO, as shown by the change in CD signals at 291 nm in Fig. 2B. In both cases, a two-state transition was observed, giving Cm values close to those observed in protein and flavin fluorescence studies (Table I). Thus, the observed transitions correspond to loss of the secondary and tertiary structures and reflect a disruption of the overall protein structure because of conversion of the folded protein into an unfolded state. Such a transition was observed at significantly lower denaturant concentrations for mutant than for wild-type CO.

**ANS Fluorescence**—The hydrophobic fluorescent probe ANS was used to probe the exposition of buried hydrophobic regions in CO at increasing urea concentrations. Binding of this probe to solvent-accessible clusters of non-polar side chains in proteins results in a marked increase in its fluorescence, and is accompanied by a blue-shift in its emission fluorescence spectrum. The steady-state fluorescence intensity of ANS significantly increased in the presence of both CO forms in their native state, reaching similar values, indicating that similar hydrophobic patches are exposed in both proteins in native conditions.

The exposition of hydrophobic regions upon loosening of the protein tertiary structure during the unfolding process was investigate by following the binding of ANS to the CO forms at increasing urea concentrations. At first, both proteins were titrated with ANS in the presence of various urea concentrations, allowing determination of the change in fluorescence emission intensity at saturating ANS concentration (ΔF), the apparent Kd for ANS binding, and the ratio between these two parameters (ΔF/Kd) (24). Values of the apparent Kd for ANS binding to wild-type CO at increasing urea concentrations remained quite constant (153 ± 20 μM), whereas the parameter ΔF/Kd varied with the urea concentration, attaining a maximum at 4 M urea (Fig. 3, A and C). These data suggest that additional ANS-accessible hydrophobic regions were exposed in wild-type CO at 4 M urea.

Different results were obtained with H69A CO. At urea concentrations up to 2 M the Kd value for ANS binding decreased significantly (from 93 to 24 μM, respectively), with a concomitant and significant increase in fluorescence emission (Fig. 3B). A further increase in urea concentrations resulted in a decrease in Kd and in an increase in ΔF. At 8 M urea both parameters reached values similar to those of wild-type CO (Kd = 120 μM and ΔF/Kd = 0.71, Fig. 3). This result indicates that the unfolding intermediate observed for the H69A mutant at ~2 M urea exposes a significantly higher hydrophobic surface with respect to wild-type CO. ANS-accessible hydrophobic surfaces were nearly identical in the fully unfolded state of either protein (i.e., at 8 M urea).

In another set of experiments, the intensity of ANS fluoresc-
Unfolding of Cholesterol Oxidase

Enzymatic Activity—Protein activity is considered a sensitive probe for studying the changes in protein conformation during denaturation, as it reflects even the subtlest readjustments at or near the active site. The effect of urea on the activity of CO was investigated by measuring the enzymatic activity on protein samples previously incubated for 60–90 min (that is, the time required to complete fluorescence changes, see above) at 15 °C in the presence of different denaturant concentrations. As reported in Fig. 1C, the enzyme activity of wild-type CO started to decline at >2.5 mM urea and is lost at 5.5 mM urea, whereas the activity of the mutant protein was already totally lost at 3 mM urea. The $C_m$ values determined from activity measurements were lower than that determined for fluorescence transitions (Table I) and were close to those observed for the first transition in ANS binding studies. These results demonstrate that the loss of catalytic activity occurs under conditions preceding the conversion of the native to the fully unfolded form of both enzyme forms (compare Figs. 1–3 and Table I).

Aggregation State—The elution volume of wild-type CO in gel-permeation chromatography decreased at increasing denaturant concentrations. The protein eluted as a monomeric species in the absence of urea ($K_w = 0.47$), as an expanded monomer at 2 mM urea ($K_w = 0.44$), as a mixture of dimeric and hexameric forms at 4 mM urea ($K_w = 0.40$ and 0.26), and as higher-order aggregates at 6 or 8 mM urea ($K_w = 0.17$ and 0.13, respectively). Therefore, the intermediate state inferred from fluorescence and CD spectroscopy at ~4 mM urea may represent an assortment of aggregates. These aggregates had a lower solubility than either native or fully unfolded CO, as made evident by the decrease in the overall peak area, that at 4 mM urea was ~65% of that measured at 0 or 8 mM urea.

The H69A mutant in the absence of urea had an elution volume similar to that of wild-type CO ($K_w = 0.46$), whereas at 2 mM urea it eluted as a mixture of dimeric and octameric forms ($K_w = 0.42$ and 0.22), and as a higher-order aggregate ($K_w = 0.17$) at ≥4 mM urea. Interestingly, the solubility of the H69A mutant at the urea concentration corresponding to the formation of the folding intermediate made evident by ANS binding experiments (~2 mM) was significantly decreased compared with the protein in either the native or fully unfolded form (~20% of protein recovered from chromatography). As expected for the mutant enzyme, and differently from wild-type CO, at urea concentrations ≥2 mM, a peak containing the released FAD cofactor was observed at high elution volume.

Reversibility of the Unfolding Process—The refolding of urea-denatured wild-type and H69A CO was studied by monitoring the recovery of enzymatic activity and the time course of changes in tryptophan and flavin fluorescence following a 10-fold dilution in plain buffer of the urea-treated proteins (initial urea concentration, 1.5–8 M). For both proteins, a large part of the changes in tryptophan and flavin fluorescence intensity upon denaturant dilution was observed in the dead-time of the experiment, followed by slower and smaller changes. In particular, the return to tryptophan fluorescence values appropriate for the urea concentration present after dilution took about 24 h for both CO forms.

The largest part of the decrease in flavin fluorescence following denaturant dilution also was observed during the experimental dead-time, and it was not observable even in rapid reaction experiments performed using a stopped-flow spectrofluorometer, indicating that this step is faster than the mixing dead-time (~2 ms). However, the fluorescence values reached at ≥24 h after urea dilution were higher than those
observed during protein unfolding experiments at the same urea concentration. This indicates that re-establishing the proper protein-cofactor interaction during the refolding process was somewhat impaired. Noteworthy, the same results were also recently observed in the case of n-amino acid oxidase, a FAD-containing flavoprotein carrying a non-covalently bound flavin (24).

Both CO enzymes recovered ~25% of the initial specific activity after unfolding with a concentration of urea at which >90% of the initial activity was lost (5 and 2.5 M, respectively, for wild-type and H69A CO), and refolding by 10-fold dilution and incubation for 24 h at 15 °C. No recovery of the enzyme activity was observed when starting from fully denatured (8 M urea) wild-type and H69A CO.

All together, these results show that the urea-induced partial unfolding of wild-type and H69A CO is reversible, at least in terms of their overall tertiary structure, but the native and refolded COs differ in the microenvironment surrounding the flavin cofactor, which in turn strongly affects their enzymatic activity.

Probing the Conformation by Limited Proteolysis

Limited proteolysis experiments have been performed to have further insights on the conformational changes ensuing from the elimination of the covalent link between the polypeptide and the flavin. SDS-PAGE analysis of the time course of proteolysis of native wild-type and H69A CO using 10% (w/w) trypsin showed that both enzyme forms were converted into two proteolytic products of ~34 and ~29 kDa. The N-terminal sequence of the 34-kDa fragment corresponded to that of intact CO starting at Ser8, whereas the sequence of the 29-kDa fragment starts at Gln105. The molecular mass of the 34-kDa band was in good agreement with the theoretical value for the Ser8-Arg104 N-terminal polypeptide (33515.9 Da), and that of the 29-kDa band with that of the Gln105-Pro161 remaining polypeptide (28899.5 Da) (numbering refers to the mature form of the enzyme).

Proteolytic conversion was complete after 150 min for wild-type CO, whereas ~15% of intact H69A CO was present after 360 min. With both proteins, the amount of the two proteolytic products originating from trypsin action on Arg105 decreased at incubation times ~120 min, indicating that these fragments were further proteolyzed. However, no other bands were detected on the SDS-PAGE gels, indicating that both fragments were fully degraded without accumulation of stable intermediates.

The elution volume from gel-permeation columns of the proteolyzed forms of both H69A and wild-type CO after 120 min treatment with trypsin was identical to that of the intact protein. Thus, trypsinolysis produced a nicked enzyme in which the proteolytic fragments remained bound under native conditions. The activity of H69A and wild-type CO eluted from gel permeation after proteolysis was 9 and 62% of that of the respective intact enzyme. These figures essentially match those for residual intact protein after 120 min of incubation (about 7 and 60% for wild-type and H69A CO, respectively, as detected by SDS-PAGE), indicating that the nicked form of both COs is inactive.

An inspection of the CO structure (4) shows that the site sensitive to trypsinolysis in the native enzyme is located on the protein surface and belongs to the loop following strand 12, which is a structural element of the substrate-binding domain, and is not in direct contact with the flavin cofactor. Structural flexibility in this region (showing a very high temperature factor) was considered of fundamental importance for substrate accessibility to the active site (4).

Limited proteolysis of both wild-type and H69A CO was also performed at urea concentrations at which the inferred unfolding intermediate is formed (4 and 2 M urea). Proteolysis of the partially unfolded proteins was much faster than under native conditions (e.g. H69A CO was completely cleaved in less than 5 min at 10% (w/w) trypsin). For this reason, proteolysis of unfolding intermediates of both proteins was performed at 1% (w/w) trypsin. In the presence of 4 M urea, wild-type CO was fully converted into the 34- and 29-kDa fragments in ~45 min, giving no additional proteolytic products. On the contrary, proteolysis of the H69A mutant in the presence of 2 M urea for 60 min gave six electrophoretic bands at ~54, 48, 34, 29, 15, and ~10 kDa, in addition to residual intact CO. N-terminal sequencing of the various products obtained from trypsinolysis of H69A CO indicated two specific proteolysis sites in addition to that detected at Arg104 in the native protein: cleavage at Arg46 gave fragments Ser8-Arg46 (3.3 kDa) and Gly66-Pro461 (54 kDa); cleavage at Trp originated the 14.8-Ser8-Arg46 (8.3 kDa) and Gly66-Pro461 (54 kDa); cleavage at Trp originated the 14.8-Ser8-Arg46 (8.3 kDa) and Gly66-Pro461 (54 kDa).

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Table II

| Method               | Wild-type | H69A |
|----------------------|-----------|------|
| Tm °C                |           |      |
| DSC, Tm of overall transition | 56.0  | 46.0 |
| Far-UV CD (220 nm)   | 60.5      | 48.1 |
| Near-UV CD (291 nm)  | 52.0      | 39.1 |
| Trp fluorescence     | 57.3      | 46.4 |
| FAD fluorescence     | 59.0      | 49.3 |
|ANS fluorescence      | 57.0      | 42.0 |

Midpoint transition temperatures as detected by the various spectroscopic signals, and are uncorrected for delay effects. Standard deviation was within 0.2 °C for all determined values.

Unfolding of Cholesterol Oxidase

Until otherwise indicated, all experiments were performed in 100 mM potassium phosphate, pH 7.5, at an identical heating rate (0.5 °C/min). Protein concentration was 0.1 mg/ml in all fluorescence measurements, but were higher for far-UV CD (0.25 mg/ml), near-UV CD (0.5 mg/ml), and DSC (1.5 mg/ml). When required, ANS was added at 0.5 mM final concentration. DSC measurements were performed in 100 mM Tris-HCl, pH 8.5, at a heating rate of 0.1 °C/min (see text and Fig. 5 and 6). Tm values were obtained by calculating the first derivative of the spectroscopic signals, and are uncorrected for delay effects. Standard deviation was within 0.2 °C for all determined values.

Temperature ramp and calorimetric (DSC) measurements were performed to assess the temperature sensitivity of both generic and specific structural features in wild-type and H69A CO, and to verify whether multiple unfolding intermediates also can be observed upon physical, as opposed to chemical, denaturation. Whenever possible, the spectroscopic approaches and general conditions (i.e. protein concentration, pH values, instrumental set up) were the same used for urea sensitivity studies. However, it has to be noted that all temperature-induced modifications in either protein were irreversible, making questionable the use of a standard van’t Hoff approach for assessing the thermodynamics of these transitions.

Midpoint transition temperatures as detected by the various experimental approaches were always ~10–15 °C higher for wild-type than for H69A CO (see Table II and Fig. 4). When temperature-induced loss of secondary structure was monitored following the CD signal at 220 nm, Tm figures were close to those observed for FAD release, as monitored in independent temperature-ramp fluorescence measurements. Studies on the temperature sensitivity of tryptophan fluorescence (taken as a reporter of tertiary structure modifications) confirmed the different inherent stability of the two CO forms, and gave Tm values slightly lower than those determined by follow-
ing flavin fluorescence and far-UV CD signals. Tertiary structure elements involving hydrophobic regions of the protein had a higher temperature sensitivity than all other modifications discussed above, as indicated by the \( T_m \) values measured in near-UV CD and ANS binding experiments. In these latter experiments, we noticed an increase in the 500 nm fluorescence as a function of temperature, resembling closely that observed for the near-UV CD signal at 291 nm. However, at higher temperatures, the fluorescence of ANS-protein mixtures decreased sensibly regardless of the CO form under investigation. This high temperature decrease is likely a consequence of the formation of protein aggregates, as reported in several other studies where ANS was used to monitor thermal protein unfolding (25, 26).

In some instances, measurements of the temperature dependence of spectroscopic signals were carried out over the 0.1–1.5 mg/ml range of protein concentration. Consistently with the occurrence of temperature-induced aggregation as hypothesized above, we noticed for either CO form a slight decrease in \( T_m \) values at increasing protein concentrations. Temperature-dependent aggregation phenomena were also evident in a first set of DSC measurements carried out in phosphate buffer, pH 7.5, and at a scanning rate of 0.5 °C/min, as for the spectroscopic studies discussed so far. In these experiments (Fig. 5), the endothermic unfolding transition for both proteins was superimposed to a sharp exothermic transition, indicative of aggregation phenomena (27). All the DSC transitions were irreversible under our conditions. The phenomena reported in Fig. 5 showed a highly reproducible pattern for each protein, indicating that formation of gels perturbs the measurements, as observed with other flavin oxidases (28), and again coincidentally support the formation of “adhesive” protein conformers during the unfolding of either form of CO at pH 7.5. These aggregated species apparently form regardless of the nature of the denaturing agent, as also indicated by the gel permeation studies in the presence of urea reported in a different section of this paper. The ANS binding studies reported in a previous section suggest a prominent role of exposed hydrophobic surfaces in the CO aggregation process, as observed for other proteins (25, 26).

Gelling phenomena in DSC were controlled by raising the pH to 8.5, where both CO forms retain full catalytic activity. At pH 8.5 the aggregation phenomena discussed above occur in a distinct (and much higher) temperature range with respect to the unfolding events, as shown in Fig. 5 for both forms of CO. The shift of aggregation phenomena toward higher temperatures at increasing pH was less pronounced for wild-type CO (from 57 to 67 °C, as compared with 53 to 75 °C for the H69A mutant). For both proteins, the shift from pH 7.5 to 8.5 did not affect the position of the endothermic events (i.e., the unfolding of the protein), or their irreversibility. This also circumstantially indicates that \( \Delta C_p \) for these proteins is negligible.

However, even data collected at pH 8.5 could not be fitted to a thermodynamic model based on equilibrium conformational transitions, indicating that concomitant irreversible events were still occurring. The kinetic effects were minimized by lowering the scanning rate to 0.1 °C/min, resulting in the thermograms shown in Fig. 5C, which provide immediate visual evidence for the different thermal stability of the two proteins. Apparent enthalpy denaturations were close for wild-type and mutant CO (1450 and 1120 kJ/mol, respectively), but \( T_d \) values...
were much lower for the H69A than for wild-type CO (46 versus 56 °C). These figures indicate that different stability of the two proteins is predominantly because of entropic factors.

In the case of wild-type CO, irreversible phenomena were not completely removed even at 0.1 °C/min, as also confirmed by a thermodynamic analysis. On the contrary, the tracings obtained for the H69A mutant were successfully and consistently analyzed thermodynamically. The results of the thermodynamic analysis lead to a model (Fig. 6) in which thermal denaturation may only be described because of three independent equilibrium transitions (29, 30). The fitting parameters, \( T_d \) and \( \Delta H \), for individual transitions were: 39.8 °C and 345 kJ/mol; 44.7 °C and 470 kJ/mol; 48.7 °C and 585 kJ/mol, for the first, second, and third transitions, respectively.

In the case of the mutant protein, where it was possible to carry out a detailed thermodynamic analysis of the independent events leading to denaturation, the final event in thermal denaturation (which is also contributing the highest enthalpy) had a midpoint temperature coinciding with the loss of secondary structure and cofactor exposure/release (Table II), as observed for other flavin oxidases (28). Also, the thermodynamic parameters associated with this transition were very close to those determined for the loss of structural elements that accompanies the release of non-covalently bound flavin in a monomeric, non-temperature-aggregating mutant of a yeast D-amino acid oxidase (39.8 and 44.7 °C for the yeast enzyme, versus 585 kJ/mol and 48.7 °C for H69A CO) (28).

Changes in tertiary structure involving hydrophobic regions of the protein may be associated to events occurring at lower temperatures in H69A CO. In particular, there is a striking coincidence between the \( T_d \) for the two low-temperature DSC-detectable transitions (39.8 and 44.7 °C) and the \( T_m \) values for loss of elements of tertiary structure and for transient exposure of surface hydrophobic sites, measured spectroscopically (see Table II).

Although a detailed thermodynamic analysis of the DSC tracings for wild-type CO was not attempted for the reasons given above, the data in Table II make it evident that the temperature sensitivity of individual events in this case follows the same order found for the mutant protein, but these events occur in a narrower temperature range in the wild-type protein (7 versus 10.3 °C, see Table II). This indicates both a higher compactness of the wild-type protein, and a higher degree of co-operativity in its temperature-induced transitions.

**DISCUSSION**

**Features of the Native Proteins**—The two CO forms differ in the visible absorption spectrum (12), as well as in their tryptophan and flavin fluorescence, and in their near-UV CD spectra. However, far-UV CD spectral properties are similar in wild-type and H69A CO. This, along with the similar sensitivity to limited proteolysis, and the very similar values of denaturation enthalpy, indicates that wild-type and H69A CO do not show significant differences in their overall topology. The spectral differences in the native proteins may be indicative of changes in the microenvironment surrounding the flavin cofactor following the deletion of the covalent link to His69, as indicated by the reported shift in the redox potential of the cofactor, that in turn affects the catalytic activity (12).

The **Unfolding Process of Wild-type and H69A CO**—The most evident conclusion drawn from the present study is that the covalent link of the flavin cofactor to the apoprotein significantly increases the structural stability of CO toward chemical and thermal denaturation. All our equilibrium spectroscopic measurements showed that the urea concentration required for the unfolding of the CO mutant containing non-covalent FAD was lower than that of the wild-type enzyme, and that different urea concentrations were required for complete unfolding of each protein. The two proteins also showed a ~10 °C difference in the midpoint temperature of individual steps leading to their thermal denaturation. Comparative analysis of the changes in the structural and functional properties of the two CO forms upon addition of urea or thermal treatment demonstrated a very good inverse correlation between the loss of “rigidity” (consequent to the elimination of the covalent link between the flavin cofactor and the polypeptide) and protein stability.

The urea-induced unfolding of both wild-type and H69A CO can be considered as a two-step (three-state) process. The presence of intermediates in the unfolding process was inferred by the different urea sensitivity of the various protein features used to monitor the folding status of CO. Urea-dependent changes in emission wavelength and intensity of tryptophan and ANS fluorescence were directly indicative of a three-state process, as were the plurality of transitions made evident by thermal denaturation spectroscopic studies, and the evidence gathered by DSC. Full unfolding of both COs was observed at urea concentrations ≥6 M, at which all the observed changes were complete and essentially irreversible, as were temperature-induced modifications, at least above 55–65 °C.

From a mechanistic standpoint, two major events occur as the urea concentration is increased: alteration of the tertiary structure and exposition of hydrophobic regions at low urea concentrations, followed by complete unfolding concomitantly to full exposition of the cofactor at higher urea concentrations. The deviation from a classical two-state behavior does not arise from the different links of the cofactor in wild-type and H69A COs, because similar unfolding profiles have been observed using both proteins (although at lower urea concentrations or at lower temperatures for the mutant CO). For a series of single-point mutants of a given protein, and provided that the parameter “m” is the same for all the mutants in the series, the \( C_m \) values determined in urea-titration experiments are proportional to the standard free energies of stabilization of the native state. In general, “m” should not change as a result of a single mutation because this parameter is interpreted as a measure of the difference between the numbers of urea interaction sites in the native and denatured states. This is not the situation with the H69A mutation of CO, indicating that the mutation altered the overall protein structure to a larger extent. This is also suggested by the extended temperature range of the different thermal unfolding steps in the mutant protein, when compared with wild-type CO. We consider the possibility that the intermediate form produced by urea has some similarity to a classical molten globule, that is, to a partially unfolded form with retained secondary structure but not regular
tertiary structure, in which extensive hydrophobic surfaces are exposed to solvent (31).

The formation of intermediate species during the unfolding process was better appreciated in the H69A mutant. Chemical denaturation experiments show that H69A CO lost its enzymatic activity and part of its characteristic tertiary structure at 2 M urea, where most of the secondary structure and non-covalent FAD binding were almost unaffected. ANS binding studies and DSC data indicate that the unfolding intermediate had exposed new hydrophobic patches on ANS-accessible surface sites that were responsible for aggregation of the partially unfolded protein. The folding intermediate(s) formed at 2 M urea shared most of the spectroscopic features found in H69A CO at subdenaturing temperatures (that is, between 42 and 46 °C).

Formation of the unfolding intermediate observed in chemical denaturation experiments for both CO forms is a reversible process, as inferred from activity recovery data, whereas no activity can be recovered from fully denatured proteins. However, minor alterations in the flavin binding region and the partial recovery of the enzyme activity are indicative of minor structural differences between the native and refolded proteins. All the unfolding/refolding data, and the dependence of DSC-detectable aggregation phenomena on the heating rate, indicate that the multiple events occurring during unfolding are kinetically, rather than thermodynamically, controlled.

Under conditions corresponding to the formation of the inferred unfolding intermediate and to the (reversible) loss of the enzymatic activity, the solubility of both CO forms was significantly impaired, suggesting that the intermediate consists of aggregated molten-globule-like proteins. An expansion of the protein before complete denaturation has been reported for several multimeric enzymes (see Ref. 32 and references therein) and for a number of other proteins (25, 33). The aggregation behavior of these “expanded” proteins may be consequent to the exposure of “sticky” surface hydrophobic sites/patches, as detected by ANS binding studies, that favor hydrophobic interaction between surfaces belonging to distinct proteins. At increasing urea concentrations, the chaotrope effect of urea disrupts the solvating water structure and leads to an expanded, fully unfolded, and soluble multimeric protein aggregate. On the contrary, temperature favors irreversible aggregation of the fully denatured species, as reported for other proteins (25, 33).

Structural Analysis—The gene encoding the CO studied here originates a monomeric polypeptide of 613 amino acids, from which an N-terminal hydrophobic pre-sequence of 52 residues is cleaved to give a 561-amino acid (62-kDa) mature form of the enzyme. During the cloning procedure, a 5-residue tag was added at the N terminus of the mature form, so that the purified enzyme constituted 569 amino acids. Its structure is composed of 12 α-helices and 17 β-strands and can be subdivided into two domains (4): a FAD-binding domain and a substrate-binding domain. The fold of the FAD-binding domain resembles that of a recently discovered family of structurally related oxidoreductases, which paradigm is represented by vanillyl-alcohol oxidase (11). The substrate-binding domain appears to be specific for CO, because it involves structural elements not present in other flavoenzymes. The active site of CO is a large cavity (with a volume of 450 Å\(^3\)) delimited by the isoalloxazine ring of the cofactor on one side and by a β-pleated sheet of the substrate-binding domain on the opposite side. Two loops in the substrate-binding domain (between β12 and α5 and between α7 and β14) show very high temperature factors and have been proposed to act as the active site entrance. The site of proteolysis under native conditions at Arg\(^{204}\) is located on the first of these two loops. The evidence presented in this study indicates that the loop following β-strand 12 of the substrate-binding domain is the region specifically more expanded in both wild-type and H69A protein forms (it is far away from the flavin-binding domain), and that the overall protein conformation is largely maintained following the elimination of the covalent link with flavin cofactor.

The pattern of proteolysis for wild-type CO was unchanged at 4 M urea, a denaturant concentration at which the conformation of the protein should largely correspond to that of the unfolding intermediate discussed in the previous section. On the contrary, the unfolding intermediate of H69A CO formed at 2 M urea showed two additional sites of proteolysis, both belonging to the flavin-binding domain. The Arg\(^{266}\) site is located at the beginning of the PP loop that also contains the site of flavinylation. Residue Gly\(^{256}\) is involved in hydrogen bond contact with the pyrophosphate group of the cofactor, as also observed for vanillyl-alcohol oxidase (11). The Trp\(^{130}\) site is also part of the flavin-binding domain and is located on the loop connecting α-helices 2 and 3. The conformation of these regions, as well as the corresponding B-factor, is not modified in native wild-type and H69A CO.

Conclusions—In conclusion, the flavin linkage to the protein moiety in CO may be regarded as a structural device that allows stabilization of the overall tertiary structure of the protein by preventing the widening of the structural gap between the two domains, at least until the whole protein structure “snaps” as a result of increasing denaturing pressure, be that chemical or physical. In the absence of this covalent link, the whole denaturation process is less co-operative and occurs at lower temperature and urea concentrations. This is made evident by the broader temperature interval for thermal denaturation of the H69A mutant versus wild-type CO, and by the presence of additional proteolysis sites in the unfolding intermediate of the H69A protein.

The relevance of our results may extend beyond the single case of CO, because from a structural standpoint it is a component of the vanillyl-alcohol oxidase family of flavoproteins (11). Thus, investigating the folding process of CO should also give a clue as for the rules that govern the folding of other covalent flavoproteins.

Recent experimental evidence demonstrated that COs bearing a covalently linked cofactor are catabolic enzymes involved in the bacterial utilization of cholesterol, whereas COs containing non-covalently bound FAD are involved in bacterial infections. In this regard, the covalent flavin 8α-N(3)histidine bond in B. stercolicum CO is an important factor in what: (a) it modulates the redox properties of the protein toward increasing its oxidative ability (12), and thus its capacity to alter the structure of the membrane in the target cells; (b) it results in a marked increase of its stability.

Besides the academic interest concerning the structure-function relationships in flavoenzymes, knowledge about protein stability is nowadays also being exploited in many practical applications in biotechnology. The CO-catalyzed reaction is already extensively employed in a broad array of applications, and a closer look at the structural determinants of the stability of these enzymes has immediate practical significance, also from an economic standpoint.

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