Relevance of the flavin binding to the stability and folding of engineered cholesterol oxidase containing noncovalently bound FAD

LAURA CALDINELLI,¹ STEFANIA IAMETTI,² ALBERTO BARBIROLI,²
DIMITRIOS FESSAS,³ FRANCESCO BONOMI,² LUCIANO PIUBELLI,¹
GIANLUCA MOLLA,¹ AND LOREDANO POLLEGIONI¹

¹Department of Biotechnology and Molecular Sciences, University of Insubria, 21100 Varese, Italy

²DISMA, University of Milan, 20133 Milan, Italy

³DISTAM, University of Milan, 20133 Milan, Italy

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Abstract

The flavoprotein cholesterol oxidase (CO) from *Brevibacterium sterolicum* is a monomeric flavoenzyme containing one molecule of FAD cofactor covalently linked to His69. The elimination of the covalent link following the His69Ala substitution was demonstrated to result in a significant decrease in activity, in the midpoint redox potential of the flavin, and in stability with respect to the wild-type enzyme, but does not modify the overall structure of the enzyme. We used CO as a model system to dissect the changes due to the elimination of the covalent link between the flavin and the protein (by comparing the wild-type and H69A CO holoproteins) with those due to the elimination of the cofactor (by comparing the holo- and apoprotein forms of H69A CO). The apoprotein of H69A CO lacks the characteristic tertiary structure of the holoprotein and displays larger hydrophobic surfaces; its urea-induced unfolding does not occur by a simple two-state mechanism and is largely nonreversible. Minor alterations in the flavin binding region are evident between the native and the refolded proteins, and are likely responsible for the low refolding yield observed. A model for the equilibrium unfolding of H69A CO that also takes into consideration the effects of cofactor binding and dissociation, and thus may be of general significance in terms of the relationships between cofactor uptake and folding in flavoproteins, is presented.

Keywords: conformational change; flavoprotein; cofactor binding; mutagenesis; fluorescence

Many proteins in nature require the binding of cofactors to perform their biological function. These molecules often fold *in vivo* in cellular environments where their cognate cofactors are present, and thus may bind to the appropriate polypeptide before folding. It is possible that local nonrandom structures form in an unfolded polypeptide due to coordination of a cofactor, and that such structural restriction(s) may make easier the conforma-

tional search for the native state. In other words, the cofactor serves as a nucleation site that drives or facilitates the overall folding process. A number of investigations (e.g., on 6-hydroxy-D-nicotine oxidase) suggest a post-translational rather than cotranslational process of flavinylation. The experiments on monoamine oxidase A and B support the view that the mechanism for covalent flavin incorporation occurs via a iminoquinone-methide addition mechanism with flavin binding preceding covalent attachment, which is facilitated by the binding of the cofactor to a presumably native-like structure of the apoprotein (for a review, see Edmondson and Newton-Vinson 2001). The observation that for a number of covalent flavoproteins (such as cholesterol oxidase) the

Reprint requests to: Loredano Pollegioni, Department of Biotechnology and Molecular Sciences, University of Insubria, via J.H. Dunant 3, 21100 Varese, Italy; e-mail: loredano.pollegioni@uninsubria.it; fax: 0332-421500.

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flavin coenzyme addition to the apoprotein allows the formation of a catalytically competent holoenzyme without covalent incorporation contrasts with a “simple” view of an autocatalytic mechanism. Present knowledge indicates that specific interactions of FAD with residues other than the site of covalent attachment are required for a covalent link, and that this interaction is productive starting from an intermediate during apoprotein folding. Also, bound cofactors almost invariably stabilize the native state of the proteins they interact with (Mewies et al. 1998).

To address directly the role of the FAD in flavoprotein folding and stability, we have examined the consequences of elimination of the covalent link between the cofactor and the protein moiety on the unfolding and FAD binding processes in *Brevibacterium sterolicum* cholesterol oxidase (CO, EC 1.1.3.6). This enzyme is an alcohol dehydrogenase/oxidase flavoprotein that catalyzes the dehydrogenation of C(3)-OH of a cholestane system to yield the corresponding carbonyl product (Gadda et al. 1997; Pollegioni et al. 1999; Sampson and Vrielink 2003). During the reductive half-reaction, the oxidized FAD cofactor accepts a hydride from the substrate, and in the ensuing oxidative half-reaction the reduced flavin transfers the redox equivalents to molecular oxygen; the enzyme also catalyzes the isomerization of cholest-5-en-3-one to cholest-4-en-3-one. COs are a group of enzymes isolated from, and frequently secreted by, a variety of microorganisms. The particular CO we studied (a prototype of type II COs) contains a FAD cofactor covalently bound to His69 (mature protein numbering, corresponding to His121 in the full-length polypeptide), and belongs to the family of vanillyl-alcohol oxidases (Fraaije et al. 1998) whose members possess a fold proposed to favor covalent flavinylation. In CO classified as type I (such as the one from *Streptomyces hygroscopicus*) the FAD cofactor is tightly but noncovalently bound to the enzyme. Enzymes belonging to these two classes show no significant sequence identities, possess different 3D structures, and have different kinetic mechanisms and redox potentials (Gadda et al. 1997; Pollegioni et al. 1999; Sampson and Vrielink 2003).

We previously reported that a His69Ala mutation prevents formation of the histidyl-FAD bond in CO (Motteran et al. 2001). This engineered protein retains catalytic activity and carries out the isomerization of the intermediate cholest-5-en-3-one to the final product. However, the noncovalent flavin binding results in a 35-fold decrease in catalytic turnover rate and in a much lower flavin midpoint redox potential (−204 mV vs. −101 mV for wild-type CO). Wild-type and H69A COs do not show significant differences in their overall topology (Lim et al. 2006), but the urea-induced unfolding of H69A CO occurred at significantly lower urea concentration (~2 M lower), as well as the temperature induced unfolding (~10°C–15°C lower), than wild-type enzyme. Urea denaturation of both proteins

occurs through a two-step (three-state) process, with the formation of an unfolding intermediate (especially evident for H69A CO at 2 M urea), in which catalytic activity and tertiary structure were lost, and new hydrophobic patches were exposed on the protein surface, resulting in protein aggregation (Caldinelli et al. 2005). We concluded that the covalent bond of the flavin in CO represents a structural device for modifying the flavin redox potentials and for stabilizing the protein tertiary structure.

In this work we address the effects of cofactor binding on stability, along with the effects of noncovalent cofactor binding on the folding process. In vitro studies aimed at characterizing the covalent flavin attachment process have been limited by difficulties in isolating reconstitutable apoenzymes. Several approaches have been used, e.g., the expression of the enzyme in a yeast strain auxotrophic for riboflavin or the replacement of crucial residues by site-directed mutagenesis (for a review, see Hefti et al. 2003). By comparing the holo- and the apo-H69A CO, we have now the opportunity to dissect the effects due to the flavin binding to an apoprotein from those due to the covalent link of the flavin cofactor (by comparing the holoenzyme forms of wild-type and H69A CO) using a unique protein scaffold and similar experimental conditions. Our approach also involved an analysis of the effects of cofactor binding on the folding yields and rates, starting from various apo-H69A conformations.

Results

Spectral properties

The holo- and apoprotein forms of recombinant H69A CO show obvious differences in their visible absorbance spectra (Motteran et al. 2001), but some other spectral properties related to protein folding also distinguish the two CO forms. Tryptophan fluorescence (following excitation at 280 nm or at 298 nm) shows an emission maximum at 333 nm, and at 338 nm for holo- and apo-H69A CO, respectively. Emission intensity is threefold higher in apo-H69A CO than in the holoprotein, indicating a different relevance of quenching interactions between tryptophans and nearby side chains in the two forms or between Trp28 and the neighboring flavin ring (Fig. 1A). For both protein forms, unfolding by urea is accompanied by a shift of the emission maximum at 354 nm and by an increase in the intensity of tryptophan fluorescence, which reaches similar values in the two fully unfolded proteins (see below).

The far-UV CD spectrum of apo-H69A CO is virtually indistinguishable from that of the holoprotein, revealing a substantial amount of secondary structure (Fig. 1B, inset). Differences are evident in the near-UV CD spectra of the two protein forms (Fig. 1B), pointing to a significant alteration of the tertiary structure in apo-H69A CO, as

Figure 1. Comparison of spectral properties of holoenzyme (solid line) and apoprotein (dashed line) of H69A CO. (A) Protein fluorescence (excitation at 280 nm, 0.1 mg protein/mL); (B) Near-UV CD spectrum (0.5 mg protein/mL). (Inset) Far-UV CD spectrum (0.2 mg protein/mL); (C) ANS fluorescence (excitation at 370 nm; 1.6 μ M protein [0.1 mg/mL] added of 560 μ M ANS). Proteins were in 50 mM potassium phosphate, pH 7.5; measurements were performed at 15°C.

also indicated by the protein fluorescence data (see above). The far- and near-UV CD features of both CO forms were abolished at ≥ 7 M urea (see below).

The hydrophobic fluorescent probe ANS was used to investigate the exposure of hydrophobic regions. Binding of this probe to solvent-accessible clusters of nonpolar side chains in proteins results in a marked increase in its fluorescence, and by a blueshift in its emission fluorescence maximum. As shown in Figure 1C, at saturating and comparable probe concentration, ANS fluorescence intensity was threefold higher in the apoprotein than in the holoprotein, being much more redshifted with this latter (maximum at 516 nm vs. 492 nm for apo-H69A CO), indicating that larger hydrophobic surfaces are exposed in the apoprotein form in native conditions.

Equilibrium unfolding studies

The intrinsic fluorescence of tryptophan residues was used to monitor protein unfolding. At increasing urea

concentration, the holo- and the apoprotein forms of H69A CO show a similar increase in emission intensity, that was in both cases complete at 7 M urea (Fig. 2A). Addition of urea also caused the emission maximum of tryptophan fluorescence of H69A CO to shift from ~ 334 nm (holoprotein) and 338 nm (apoprotein) to 354 nm at >6 M urea. In both protein forms this wavelength shift anticipated the change in fluorescence intensity (Fig. 2B). A fluorescence redshift stems from the transfer of tryptophan side chains to a more polar environment upon protein unfolding (Burstein et al. 1973).

When urea-induced loss of secondary structure elements was monitored by following the changes in ellipticity at 220 nm, the CD signal of holo- and apoprotein H69A CO showed a similar sensitivity to the denaturant (C_m of 3.7 and 3.5 M, respectively), although the change was less cooperative in the absence of the cofactor (see Table 1). Addition of urea had a similar effect on the tertiary structure in the case of the holoenzyme, whereas for the H69A CO apoprotein it was

Figure 2. Equilibrium denaturation curves of holo- (circles, solid lines) and apoprotein (squares, dashed lines) of H69A CO detected by using different techniques. (A,B) Tryptophan fluorescence (excitation at 280 nm, 0.1 mg protein/mL): The fraction of unfolded CO was determined from the fluorescence intensity at ~ 340 nm using the value for the untreated and 8-M urea-treated proteins as reference (A); position of the emission peak maximum (B). (C) Near-UV CD measurements at 291 nm (proteins were 0.5 mg/mL). Lines represent the best fit obtained using a two-state denaturation model (Tanford 1968; Pace 1990; Caldinelli et al. 2005). For all experiments, CO samples were equilibrated at 15°C in 50 mM potassium phosphate, pH 7.5, containing the given urea concentration. The reported values have been corrected for readings prior to protein addition.

Table 1. Comparison of thermodynamic parameters for urea-induced unfolding of holo- and apoprotein of H69A COs at 15°C, monitored by using different spectral probes

	H69A holoenzyme ^a				H69A apoprotein		
	Fluorescence		CD		Fluorescence	CD	
	exc 280 nm	exc 450 nm	Near	Far	exc 280 nm	Near	Far
ΔG°_w (kJ/mol)	6.4	5.7	15.6	10.6	5.0	13.5	8.3
m (kJ L/mol ²)	2.1	1.6	4.3	3.0	1.8	6.3	2.2
C_m (M)	2.9 (1.9) – 1.7 ^b		3.6	3.5	2.7 (2.4)	2.2	3.7

Protein samples were in 50 mM potassium phosphate, pH 7.5, at the following concentrations: fluorescence measurements, 0.02 mg/mL; near-UV CD, 0.5 mg/mL; far-UV CD 0.25 mg/mL. The C_m value determined from the change in the wavelength of fluorescence emission maximum is reported in parentheses.

The difference in free energy between the folded and the unfolded state ΔG , was determined from the graphs such as those in Figure 2, assuming that it is largely dependent on the urea concentration, and the following equation: $\Delta G^\circ = \Delta G^\circ_w - m(C_m - C)$.

^aCaldinelli et al. 2005.

^bValue determined from the loss of catalytic activity.

significantly more chaotrope-sensitive, as shown by the CD changes at 291 nm (Fig. 2C).

A two-state mechanism of unfolding, in which only native and denatured molecules are taken into account (Pace 1990) fits excellently to the unfolding data. The free energy of unfolding, ΔG° , was calculated as reported previously (Caldinelli et al. 2005). The free energy of unfolding in the absence of the denaturant, ΔG°_w , the slope of the unfolding curve, m , and the midpoint concentration of urea required for unfolding, C_m , are reported in Table 1 for the holo- and apoprotein form of H69A CO.

In the case of the H69A holoenzyme, similar C_m values were derived from fluorescence and CD signals (in the 2.9–3.5 M range), but not for the protein fluorescence emission maximum. The same transitions were observed for the apoprotein form at lower C_m values (in the 2.2–2.7 M range), although changes in secondary structure in this case showed a significantly higher C_m (3.7 M, Table 1). Importantly, the transitions corresponding to the loss of secondary structure elements showed the same behavior for the holo- and apo-H69A CO.

As a further marker of changes in the protein tertiary structure during the unfolding process, the exposition of hydrophobic regions was investigated by following the binding of ANS to the two H69A CO forms at increasing urea concentration. At first, both proteins were titrated with ANS in the presence of various urea concentrations, thus allowing determination of changes in fluorescence emission intensity at saturating ANS concentration (ΔF), the apparent K_d for ANS binding, and the ratio between these two parameters ($\Delta F/K_d$) (Caldinelli et al. 2004), after correction for the effect of urea on ANS fluorescence. In the case of the H69A CO holoprotein, the fluorescence intensity of protein-bound ANS reached a maximum at 2 M urea, and the K_d value decreased significantly at urea concentrations up to 2 M (from 93 to 24 μ M, respectively; Fig. 3A). In the case of the apo-

H69A, the protein-bound ANS fluorescence decreased at increasing concentration of the denaturing agent, with a concomitant increase in K_d (Fig. 3B).

Overall surface hydrophobicity, as defined by the $\Delta F/K_d$ ratio, attains a maximum at 2 M urea for the H69A CO holoprotein, but decreases at increasing urea concentrations for the apoprotein (Fig. 3C). These data suggest that

Figure 3. Results of titration of holo- (A) and apoprotein (B) of H69A CO with ANS, as a function of urea concentration. ΔF (filled symbols): maximal change in fluorescence intensity at 500 nm at saturating ANS concentration; K_d (open symbols) apparent dissociation constant for ANS binding. (C) Ratio of the ΔF and K_d values determined at different urea concentrations for the holo- (●) and apoprotein (■) of H69A, and for the wild-type CO (▲). Protein samples (0.1 mg/mL) were equilibrated for 60 min at 15°C in 50 mM potassium phosphate, pH 7.5, at the given urea concentration. Following each addition of ANS to the protein–urea mixtures, fluorescence spectra were recorded from 450 to 600 nm with excitation at 370 nm.

additional ANS-accessible hydrophobic regions were exposed in the holoenzyme at increasing urea concentration, as observed for wild-type CO, reaching a maximum at the denaturant concentration at which the unfolding intermediate is most abundant (Caldinelli et al. 2005). On the other hand, the exposure of hydrophobic regions is high for the apo-H69A in the native state, and it decreases at increasing urea concentrations. Again, as observed for all structural parameters considered so far, no differences in surface hydrophobicity parameters were evident among the various protein forms, including wild-type H69A CO (Caldinelli et al. 2005), at urea concentrations higher than 6 M.

In another set of experiments, the intensity of ANS fluorescence was measured in solutions containing fixed amounts of protein and ANS, and increasing denaturant concentration. Two transitions were evident for the H69A holoprotein: (1) Low urea concentrations gave an increase in ANS fluorescence intensity accompanied by a shift in the ANS emission wavelength maximum (from 516 nm to 482 nm at 3 M urea); (2) higher urea concentrations resulted in fluorescence quenching and in a shift in the emission wavelength maximum to ≈ 520 nm, both being complete at ≥ 6 M urea (Fig. 4A). In the case of the apo-H69A, low urea concentrations gave a decrease in ANS fluorescence intensity and a slight change in the wavelength of maximum fluorescence (Fig. 4B), whereas it behaved similarly to the holoprotein at higher urea concentrations.

All together these results indicate that additional hydrophobic ANS-binding surfaces are exposed in the course of the low-urea induced transitions only in the

H69A CO holoprotein, and that these surfaces progressively disappeared in both the holo- and apoprotein forms of H69A CO as the urea concentration was increased further.

Spectroscopic and calorimetric studies on the thermal stability

Temperature ramp experiments were performed to compare the temperature sensitivity of specific structural features in the holo- and apoprotein of mutant H69A CO forms, and to assess the nature and extent of spectroscopic modifications ensuing by progressive heating. Conditions used when monitoring temperature-dependent changes of the various spectroscopic features of the protein were identical to those used in DSC experiments. Studies on the temperature sensitivity of tryptophan fluorescence (taken as a reporter of tertiary structure modifications), of ANS fluorescence, and of the CD signal at 225 nm (taken as a reporter of the change in secondary structure) show the different inherent stability of the holo- and apo-H69A proteins (Fig. 5A–C). The presence of aggregation-association phenomena before the completion of the denaturation process at 0.5 mg/mL apoprotein concentration made it difficult to assess the temperature sensitivity of near-UV CD signals at 291 nm.

Midpoint transition temperatures as detected by the various experimental approaches were always $\sim 7^\circ\text{C}$ – 10°C higher for the holo- than for the apoprotein of H69A CO (see Table 2; Fig. 5). In the case of the holoprotein, tertiary structure elements involving hydrophobic regions of the protein showed 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. For all the CO forms under investigations, the fluorescence of ANS–protein mixtures decreased sensibly at higher temperatures as a consequence of the formation of protein aggregates, as reported in several other studies where ANS was used to monitor thermal protein unfolding (Iametti et al. 1996; Pollegioni et al. 2003; Caldinelli et al. 2005).

The T_m values determined by monitoring the different spectroscopic signals were significantly closer for the H69A CO apoprotein than for the holoprotein. These data suggest that apo-H69A CO does not benefit—as does the holoprotein—from interactions capable of stabilizing structural regions toward temperature-induced denaturation. This hypothesis is supported by DSC studies (Fig. 5B, inset), in which a single weak signal ($\Delta H = 270 \pm 30 \text{ kJ mol}^{-1}$, almost fitting to a single state transition) was observed ($T_d = 35.5 \pm 0.3^\circ\text{C}$). This is very different from that observed in DSC studies on the wild-type and H69A CO holoproteins, both showing multiple thermodynamically independent and much less temperature-sensitive domains

Figure 4. Effect of urea on the ANS fluorescence in the presence of holo- (A, circles) and apoprotein (B, squares) of H69A CO. Fluorescence intensity at 500 nm (filled symbols) and emission maximum (open symbols) were determined on CO samples (0.1 mg protein/mL) equilibrated for 60 min at 15°C in the presence of increasing urea concentrations in 50 mM potassium phosphate, pH 7.5. Fluorescence spectra were recorded with excitation at 370 nm after the addition of 0.1 mM ANS. The reported values have been corrected for the emission of the solution prior to ANS addition.

Figure 5. Temperature dependence of spectroscopic signals for the holo- (●) and apoprotein (■) of H69A CO: (A) tryptophan fluorescence; (B) ANS fluorescence; (C) far-UV CD. All experiments were performed in 50 mM potassium phosphate, pH 7.5. Protein concentration was 0.1 mg/mL in all fluorescence measurements, but was higher for far-UV CD (0.25 mg/mL). When required, ANS was added at 0.5 mM final concentration. Spectral signals were monitored continuously during progressive heating from 20°C to 80°C at a heating rate of 0.5°C/min, and are given as percent of the total observed change regardless of the direction of the change. *Inset* of B: DSC trace for apo-H69A (dashed line), heating rate 0.5°C/min (a similar result was obtained at heating rate 0.1°C/min, data not shown). Protein was 1.5 mg/mL in 100 mM Tris-HCl, pH 8.5. The continuous solid line is a fit performed according to single step equilibrium transition model ($\Delta H = 270 \text{ kJ mol}^{-1}$, $T_d = 35.5^\circ\text{C}$ used as fitting parameters).

(Caldinelli et al. 2005), and confirms that the effects of flavin binding extend to the overall protein structure.

The FAD-binding process: Reconstituting differently folded apoproteins

The kinetics of reconstitution of the H69A apoprotein–FAD complex were previously studied under pseudo first-order conditions (>10-fold excess of either FAD or apoprotein), by following the time course of quenching of the protein or flavin fluorescence, as appropriate (Motteran et al. 2001). In both cases, the time course of the reaction is biphasic. The initial fast phase and the slow secondary change of the protein fluorescence following the addition of excess FAD proceeds according to a first-order exponential process. The observed first-order

rate constant of the fast phase depended linearly on the concentration of the cofactor, whereas the rate constant for the second (slow) phase was not dependent on the apoprotein concentration. The process can thus be described by two subsequent kinetic steps:

with rate constants $k_1 = 1.4 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ and $k_2 = 2 \times 10^{-4} \text{ s}^{-1}$ (in the absence of chaotropic agents, the reversal rate constants can be assumed to be close to zero).

The conformational changes of the apoprotein during the FAD-binding process were probed by limited proteolysis experiments. We previously demonstrated that wild-type and H69A CO holoenzymes are converted by trypsin cleavage into a nicked and inactive form constituted by two proteolytic fragments that remained bound under native conditions (Caldinelli et al. 2005). Under the same experimental conditions, the H69A CO apoprotein is quickly proteolyzed in ≤ 5 min at 10% (w/w) trypsin, but is largely protected from proteolysis by previous incubation for ≤ 30 s with the coenzyme. This indicates that a significant change in the apoprotein conformation is obtained within 1 min from the coenzyme addition to the nondenatured apoprotein. This corresponds to the time required to complete the first phase of cofactor reconstitution, as indicated by the fluorescence studies reported above. The sensitivity of the reconstituted holoprotein to

Table 2. Comparison of T_m as determined by different approaches on wild-type and holo- and apoprotein of H69A mutant CO

Method	T_m (°C)		
	Wild type ^a	H69A Holozyme	H69A Apoprotein
DSC, T_d of overall transition	56.0	46.0	35.5
Far-UV CD (220 nm)	60.5	48.1	~37
Near-UV CD (291 nm)	52.0	39.1	n.d.
Trp fluorescence			
(FAD fluorescence)	57.3 (59.0)	45.0 [48.1]	37.8 [37.2]
ANS fluorescence	57.0	42.2 [45.4]	35.5 [32.1]

CD and fluorescence experiments were performed in 100 mM potassium phosphate, pH 7.5, at an identical heating rate (0.5°C/min); the values determined in 100 mM Tris–HCl pH 8.5 are reported in square brackets. Protein concentration was 0.1 mg/mL in all fluorescence measurements, but it was 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. 6). T_m s 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 the values reported here.

^aCaldinelli et al. 2005.

trypsin is slightly higher than that of the native H69A holoenzyme (60% residual intact protein for the reconstituted H69A CO holoprotein vs. 80% for the native, and 0% for the apoprotein after 5 min of proteolysis).

To assess whether the reported unfolding intermediate(s) had a role in cofactor uptake, we studied the time course of FAD binding/release to the H69A CO forms in the presence and in the absence of 2 M urea. The release of FAD from the H69A CO holoprotein—as estimated from protein and flavin fluorescence changes—is a monophasic process and was significantly faster (at least 50-fold) in the presence of 2 M urea (Fig. 6A). The addition of 2.5 M KBr further increased the rate of flavin release up to 35- and 200-fold in the presence and in the absence of denaturant, respectively (see the protein fluorescence time courses reported in Fig. 6B). On the other hand, reaction rates for FAD uptake measured by following the quenching of the protein fluorescence were

Figure 6. Effect of 2 M urea on release (A,B) and binding (C) of flavin cofactor to H69A CO. (A) Time course of protein (filled symbols) and flavin (open symbols) fluorescence released by H69A CO (0.2 mg protein/mL) in the absence (circle) and in the presence (square) of 2 M urea. (B) The samples obtained as reported in (A) were incubated in a buffer containing 2.5 M KBr and the same concentration of urea as above. (C) Time course of protein fluorescence intensity at 340 nm during the FAD binding (1.3 μ M) to apo-H69A (0.12 μ M, 0.0075 mg/mL) in the absence (circle) and in the presence (square) of 2 M urea. All the experiments were performed at 15°C in 50 mM potassium phosphate, pH 7.5.

very similar at 0 and 2 M urea ($k_{\text{obs1}} = 0.0015 \pm 0.0001 \text{ s}^{-1}$ and $0.0017 \pm 0.0002 \text{ s}^{-1}$, respectively, in 0.25 M KBr and 2% glycerol; Fig. 6C).

The refolding of urea-denatured wild-type and H69A CO holoproteins were previously studied by monitoring the changes following a 10-fold dilution in plain buffer of the urea-treated proteins (Caldinelli et al. 2005). These results showed that the urea-induced partial unfolding of wild-type and H69A CO holoenzymes is for the most part reversible, at least in terms of their overall tertiary structure as inferred from flavin and protein fluorescence. Both wild-type and mutant CO recovered $\sim 25\%$ of the initial specific activity after unfolding with a concentration of urea at which $\sim 90\%$ of the initial activity is lost (5 M and 2.5 M for wild-type and H69A CO, respectively), but no recovery of the enzyme activity was observed when starting from the fully denatured form prepared by treatment with 8 M urea. The refolding of 2 M and 6 M urea-denatured apo-H69A was investigated as a function of the timing of FAD addition and of urea dilution. Structural recovery as monitored by tryptophan fluorescence was slightly higher following the addition of FAD to a 10-fold diluted denatured apoprotein (see Fig. 7). At both starting denaturant concentrations, the protein fluorescence of the reconstituted H69A CO was higher than that reached by the native holoprotein exposed to the same urea concentration, and the wavelength maximum after dilution invariably was close to that of the apoprotein form (Fig. 7). In comparison to the enzymatic activity recovered for the H69A holoprotein reconstituted in the absence of denaturant, a value in the 20%–35% range was obtained starting from 2 M- or 6 M-treated apoprotein. Yields and rates of the process were not modified by the presence of 20% glycerol as a flavoprotein chemical chaperone (Raibeikas and Massey 1997), or by the presence of an equimolar amount of GroEL (Li et al. 2006).

Discussion

Wild-type and H69A CO show only minor differences in the overall fold of the protein (Croteau and Vrieling 1996; Lim et al. 2006). However, the slight movement of two loops and a 180° rotation of Trp28 change the geometry of the isalloxazine ring of the flavin from non-planar to planar, with consequences on the spectroscopic properties of the flavin cofactor and on the enzyme activity (Motteran et al. 2001). Thus, subtle differences in tertiary structure, localized in the flavin-harboring domain and related to the presence/absence of a covalent bond, dictate the structural and functional behavior of the protein. As indicated by our Far-UV CD data, the apo-H69A CO retains most of the holoprotein secondary structure. However, apo-H69A shows a conformation characterized by a larger exposure of hydrophobic surfaces, a higher protein

Figure 7. Comparison of the protein fluorescence spectrum of holo- (1) and apoprotein (2) of H69A CO with that obtained following the reconstitution of apoprotein with FAD (3,4). Reconstituted H69A CO holoenzyme obtained starting from 6 M urea-treated apoprotein and addition of 10-molar excess of FAD simultaneously to 10-fold dilution of denaturant (3) or after the 10-fold dilution of denaturant for 1 h at 15°C (4).

fluorescence (that could be attributed to Trp28), and a higher sensitivity to proteolytic cleavage, with respect to the CO holoprotein. Limited proteolysis data demonstrated that FAD binding to the apo-H69A very rapidly produces (<1 min) a compact conformation, very similar to that of the H69A CO holoenzyme.

The most evident conclusion from the present study is that binding of the flavin cofactor significantly increases the structural stability of H69A CO toward chemical and thermal denaturation. All the equilibrium spectroscopic measurements showed that the urea concentration required for the unfolding of the apo-H69A CO was lower than that of the holoprotein (see Table 1). The main

exception is the structural transition corresponding to loss of the secondary structure that was observed at similar denaturant concentration for either form of H69A CO. Loss of secondary structure elements was found to coincide with FAD release from the H69A CO holoprotein. Also in wild-type CO, in which the cofactor is covalently linked, the loss of secondary structure occurred at chaotrope concentrations where the fluorescence of the covalently bound cofactor became identical to that of free FAD (Caldinelli et al. 2005). As reported there, the urea sensitivity of these features was much lower in the wild-type CO than in the H69A mutant.

The urea-induced unfolding process of both holo- and apoprotein of H69A CO can be considered as a two-step (three-state) process, analogously to that reported for the wild-type enzyme (Caldinelli et al. 2005). The presence of intermediates in the unfolding process can be principally inferred by the different urea sensitivity of the changes in emission wavelength and intensity of tryptophan fluorescence, and in ANS fluorescence. A three-state process for the unfolding of the apoprotein form of H69A CO is also suggested by the higher C_m value observed in far-UV CD signal compared to the other experimental structural probes/features considered in this study.

Chemical denaturation experiments also show that H69A CO produces at 2 M urea a structural intermediate devoid of enzymatic activity and of a major part of its characteristic tertiary structure. This unfolding intermediate retains most of the secondary structure and, in the case

Scheme 1. A schematic representation of the structural changes of holoenzyme and apoprotein of H69A COs ensuing from increasing concentrations of urea. The arrows indicate the sites of trypsin cleavage.

of the holoprotein, some of the regions involved in noncovalent FAD binding. The unfolding intermediate can be obtained from either the apo- or holoform of H69A CO, and shows increased ease of FAD uptake/release, as if some of the regions involved in retention of the noncovalently bound cofactor (or in its access to binding sites on the apoprotein) were characterized by a greater accessibility. In this frame, it is worth noting that both 2 M urea and 2.5 M KBr significantly increase the rate of FAD release from the H69A CO holoprotein (Fig. 6): 2.5 M KBr not only provided the required ionic strength but can be regarded itself as a mild ionic chaotrope.

For the holoenzyme forms of CO (containing both covalently and noncovalently bound FAD) two events occur as the urea concentration is increased (Caldinelli et al. 2005): at first, the alteration of the tertiary structure and exposition of hydrophobic regions, followed by complete unfolding concomitantly to full exposition of the cofactor and lack of the secondary structure at high urea concentrations. The elimination of the FAD cofactor modifies the first event since the apoprotein possesses a higher exposition of hydrophobic surfaces and a different tertiary structure compared to the holoenzyme, but even for the apoprotein form the abolition of the secondary structure (e.g., by urea or heating) is mainly responsible for full denaturation. Importantly, COs are extracellular enzymes, and little is known about how quality-control mechanism(s) actually operate in extracellular space. While not excluding other possibilities, the most considered model for extracellular quality control is based on selective endocytosis of nonnative extracellular proteins through receptors and intracellular degradation (Yerbury et al. 2005). Recent observations are consistent with the hypothesis that exposed hydrophobicity might be the structural change that identifies individual extracellular proteins as needing quality-control intervention. Further support for this hypothesis has come from the demonstration that an extracellular chaperone, clusterin, binds to its substrates by hydrophobic interactions (Poon et al. 2002). This increase in hydrophobicity is exactly what we have also observed in the CO apoprotein form and in the unfolding intermediate compared to the folded holoenzyme forms.

A model for the equilibrium unfolding of H69A CO apoprotein and holoenzyme is shown in Scheme 1. This model is based on the three-state unfolding–refolding process discussed above, and on the reversible binding of FAD to the apoprotein polypeptide under appropriate conditions, namely, before some specific and yet undefined structural elements are lost in the chaotrope-generated unfolding intermediate discussed above. The nature and location of these regions remain elusive, also because the DSC data obtained for the H69A CO apoprotein indicate that FAD removal results in a rather

broad destabilization of the overall structure, without pointing out to a specific structural region. Our activity recovery data indicate that chemical denaturation of the H69A CO apoprotein is only partially reversible even at 2 M urea, and even in the presence of the cofactor, although protein and flavin fluorescence data are indicative that structural differences between the native and the refolded/reconstituted protein are only minor. On the other hand, significantly higher refolding yields (but not a faster recovery of the catalytic properties) are observed when FAD is added to the apoprotein in the absence of a previous denaturing step.

Based on the data discussed above, and on our previous comparison between stability of the wild-type and H69A CO holoproteins (Caldinelli et al. 2005), it may be concluded that the covalent flavin linkage to the CO polypeptide stabilizes the overall tertiary structure of the protein by preventing a broadening of the structural gap between the two domains. This is made evident, among others, by the presence of additional proteolysis sites in the unfolding intermediate of the H69A CO with respect to the wild-type protein (Caldinelli et al. 2005), as well as by the ANS binding data presented here, that complement previous observations on the inverse relationship between the “solidity” of FAD binding (or the absence of bound FAD, as reported here for the H69A apoprotein) and the degree of “openness” of the tertiary structure of the protein.

In more general terms, reversibility of unfolding independent of cofactor uptake seems to be mostly a property shared by small complex proteins, independent of the organic or inorganic nature of the cofactor, and is seldom observed in large, multidomain proteins. As an example within flavoproteins, flavodoxin—a small (179 residues) protein that contains a molecule of noncovalently bound FMN—has been shown to refold reversibly after treatment with guanidinium chloride, even in the absence of the cofactor (van Mierlo et al. 1998). On the contrary, as shown by our previous studies on wild-type CO, even the presence of a covalently bound FAD is not sufficient to drive refolding of a completely denatured large multidomain protein—such as CO—into a catalytically competent species (Motteran et al. 2001; Caldinelli et al. 2005). In fact, even minor modifications in the conformation of the mature, previously folded protein can prevent the full recovery of the native conformation of CO. Differently from other covalent flavoproteins (Robinson and Lemire 1996), wild-type CO may avoid these problems *in vivo* by establishing a covalent link with the cofactor during the folding process, where the covalently bound FAD may contribute to ensure appropriate local folding and, subsequently, an appropriate set of short- and long-distance interactions within the enzyme structure as a whole. Even small alterations in tertiary structure, and specifically an increase of surface

hydrophobicity, decrease or abrogate the enzymatic activity and could target the protein toward degradation. In agreement with what was stated by Edmondson and Newton-Vinson (2001), these results contrast with the “simple view” of an autocatalytic mechanism of covalent flavin incorporation in which the apoprotein folds to a conformation competent for both flavin binding and covalent bond formation.

Furthermore, the elucidation of the specific interactions between flavoproteins and their cofactors is of main importance even from a medical point of view. In fact, mutations that affect the affinity for the flavin have been identified in human glutathione reductase (that can lead to nonspherocytic hemolytic anemia), NADPH-reductase, methylenetetrahydrofolate reductase, etc., as well as in the apoptosis inducing factor (that can activate a caspase-independent cell-death pathway) (Hefti et al. 2003). The understanding of the flavin binding process and of the role of the protein moiety in the mechanism of covalent flavinylation will facilitate the development of new drugs able to stabilize specific conformomers in the folding pathway of complex flavoproteins.

Materials and Methods

Enzymes

Wild-type and H69A recombinant COs were obtained from Roche Diagnostics. Enzyme concentration was determined by using known extinction coefficients at 445 nm ($16.1 \text{ mM}^{-1} \text{ cm}^{-1}$ and $13.4 \text{ mM}^{-1} \text{ cm}^{-1}$ for H69A and wild-type CO, respectively) (Motteran et al. 2001). Apo-H69A CO was prepared by overnight dialysis of the holoprotein against 2.5 M KBr (Motteran et al. 2001), and its concentration assessed by using an extinction coefficient of $137.6 \text{ mM}^{-1} \text{ cm}^{-1}$ at 280 nm.

Enzymatic activity

Cholesterol oxidase activity was assayed by monitoring H_2O_2 production in an enzyme-coupled assay (Gadda et al. 1997; Pollegioni et al. 1999) carried out at 25°C in 50 mM potassium phosphate buffer, pH 7.5, containing 1% (v/v) Thesit, and 1% (v/v) propan-2-ol.

Absorption and fluorescence measurements

Absorption spectra were recorded with a Jasco V-560 spectrophotometer in 50 mM potassium phosphate buffer, pH 7.5, at 25°C. Flavin and protein fluorescence measurements were performed in a Jasco FP-750 instrument equipped with a thermostated cell holder by using a 1-mL cell. Tryptophan emission spectra were taken from 300 to 400 nm with excitation at 280 or 298 nm. Flavin emission spectra were recorded from 475 to 600 nm with excitation at 450 nm and 10 nm bandwidth for both excitation and emission. Steady-state fluorescence measurements were performed at 15°C and at 0.1 mg/mL protein concentration, and corrected for buffer contributions.

Temperature-ramp fluorescence experiments were performed by using a software-driven, Peltier-based temperature controller, that allowed reproducing the same temperature gradient ($0.5^\circ\text{C}/\text{min}$) used in temperature-ramp circular dichroism measurements and in calorimetric studies. Fixed wavelength emission measurements were taken at 340 nm and 526 nm for tryptophan and flavin fluorescence, respectively. Circular dichroism (CD) spectra were recorded on a J-810 Jasco spectropolarimeter, also equipped with a software-driven Peltier-based temperature controller, and analyzed by means of Jasco software. Cell path length was 1 cm for measurements above 250 nm, and 0.1 cm for measurements in the 190–250 nm region.

Limited proteolysis

Various CO forms (0.4 mg/mL, corresponding to $\sim 6.4 \text{ }\mu\text{M}$) were incubated at 25°C in 100 mM potassium phosphate, pH 7.5, with 10% (w/w) trypsin. Protein samples (7 μg) taken at various times after trypsin addition were diluted in sample buffer for SDS-PAGE, heated at 100°C for 3 min and analyzed electrophoretically. The intensity of the protein bands was determined by densitometric analysis using the Image Master 1D software (Amersham Pharmacia Biotech). Changes in apoprotein conformation during the cofactor binding process were analyzed by starting the proteolysis reaction using 1.25 μM apoprotein samples preincubated for different times (0–120 min) with 12.6 μM FAD. Kinetic data for protein fragment formation/degradation were fit to either a single- or a double-exponential decay equation by using KaleidaGraph (Sinergy Software).

Calorimetry

Calorimetric measurements were carried out on 23 μM (1.5 mg/mL) protein in 100 mM Tris-HCl, pH 8.5, at scan rates of 0.1 and $0.5^\circ\text{C}/\text{min}$. Data were analyzed by means of the THESEUS software (Barone et al. 1992), following procedures reported in previous studies (Pollegioni et al. 2003; Caldinelli et al. 2005).

Equilibrium unfolding–refolding and ANS binding experiments

The unfolding equilibrium of apo-H69A CO was determined by following the changes in protein fluorescence, as well as in near- and far-UV CD signals, as detailed elsewhere (Caldinelli et al. 2005), by primarily using the “consensus” set of experimental conditions suggested by (Maxwell et al. 2005). Each point in urea denaturation curves was determined on an individual sample, prepared by mixing appropriate volumes of a concentrated protein, of 8 M urea in buffer, and of 50 mM potassium phosphate, pH 7.5.

ANS binding experiments were carried out at 15°C and at 0.1 mg/mL protein concentration. ANS fluorescence emission spectra were recorded in the 450–600 nm range with excitation at 370 nm. For unfolding experiments, protein samples were incubated for 60 min at 15°C in buffer containing different concentrations of urea, after which ANS was added to a final concentration of 0.1 mM.

In all cases, the unfolding curves were analyzed by fitting to a two-state mechanism as described previously (Tanford 1968; Pace 1990; Caldinelli et al. 2005). A least-squares curve fitting

analysis was used to calculate the values of ΔG_w , m , and C_m by a software routine.

To investigate flavin uptake, apo-H69A CO was incubated in the absence or in the presence of 2 M or 6 M urea for 60 min, and then refolded by 10-fold dilution in 50 mM potassium phosphate, pH 7.5 and 25°C, in the presence of a 10-fold molar excess of FAD. The refolding yield was determined by monitoring protein fluorescence and the recovery of enzymatic activity (Motteran et al. 2001). Refolding experiments were also performed by adding excess FAD after or simultaneously to denaturant dilution, or in the presence of 20% glycerol, or of a GroEL tetradecamer (Li et al. 2006) in equimolar amount with respect to apo-H69A CO.

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