Probing the role of tryptophan residues in a cellulose-binding domain by chemical modification

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Abstract

The cellulose-binding domain (CBD_{Cex}) of the mixed function glucanase–xylanase Cex from *Cellulomonas fimi* contains five tryptophans, two of which are located within the β -barrel structure and three exposed on the surface (Xu GY et al., 1995, *Biochemistry* 34:6993–7009). Although all five tryptophans can be oxidized by *N*-bromosuccinimide (NBS), stopped-flow measurements show that three tryptophans react faster than the other two. NMR analysis during the titration of CBD_{Cex} with NBS shows that the tryptophans on the surface of the protein are fully oxidized before there is significant reaction with the two buried tryptophans. Additionally, modification of the exposed tryptophans does not affect the conformation of the backbone of CBD_{Cex}, whereas complete oxidation of all five tryptophans denatures the polypeptide. The modification of the equivalent of one and two tryptophans by NBS reduces binding of CBD_{Cex} to cellulose by 70% and 90%, respectively. This confirms the direct role of the exposed aromatic residues in the binding of CBD_{Cex} to cellulose. Although adsorption to cellulose does afford some protection against NBS, as evidenced by the increased quantity of NBS required to oxidize all of the tryptophan residues, the polypeptide can still be oxidized completely when adsorbed. This suggests that, whereas the binding appears to be irreversible overall [Ong E et al., 1989, *Bio/Technology* 7:604–607], each of the exposed tryptophans interacts reversibly with cellulose.

Keywords: absorbance spectroscopy; cellulose-binding; fluorescence spectroscopy; *N*-bromosuccinimide; NMR

Many β -1,4-glycanases that hydrolyze the polysaccharides in plant cell walls have discrete cellulose-binding domains. Analysis of the adsorption of a CBD to cellulose is complicated by the nature of the matrix to which the polypeptide adsorbs. Cellulose, in effect, presents an array of multiple, overlapping binding sites to the CBD. For example, CBD_{Cex} from the xylanase–glucanase Cex of the bacterium *Cellulomonas fimi* was shown to bind cellulose within a few seconds (Gilkes et al., 1992), and it appears to bind irreversibly (Ong et al., 1989, 1991; Creagh et al., 1996). However, the

precise structural basis for the interaction of this CBD with cellulose remains to be established.

CBDs are classified into 10 families of related amino acid sequences (Tomme et al., 1995). As the number of CBDs assigned to a family increases, the number of amino acids strictly conserved in all of them decreases. These conserved amino acids are assumed to be important to the structure and function of the CBDs. The CBDs of family II, of which CBD_{Cex} is a member, are about 100 amino acids long. Recently, the three-dimensional structure of CBD_{Cex} was determined using NMR methods (Xu et al., 1995). This revealed that CBD_{Cex} is folded as a nine-stranded β -barrel with three of its five tryptophans aligned along one face of the barrel. The two other tryptophans are buried within the interior of the polypeptide (Fig. 1; Xu et al., 1995). Three of these tryptophans are conserved in all members of this family (W17, W38, and W54; Fig. 1); and a fourth is conserved in the great majority (W72; Fig. 1) (Tomme et al., 1995). Previous site-directed mutation of these tryptophans shows that, in general, their presence is required for binding. However, the results of mutations for the analogous residues among different members of family II CBDs is contradictory. For example,

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Abbreviations: BMCC, bacterial micro-crystalline cellulose; CBD, cellulose-binding domain; CBD_{Cex}, CBD from the *Cellulomonas fimi* xylanase–glucanase Cex; ($\delta^4A/\delta\lambda^4$)_{Trp}, contributions in the fourth derivative spectrum between 292 and 295 nm due to the unmodified tryptophan side chain; EGII, endoglucanase II; HSQC, heteronuclear single quantum coherence; NBS, *N*-bromosuccinimide.

Fig. 1. Ribbon diagram of the structure of CBD_{Cex} in solution indicating the β -strands and position of the Trp side chains in the molecule. The figure was created using the program MOLSCRIPT (Kraulis, 1991).

mutation of W66 (corresponding to W72 in CBD_{Cex}) in CBD_{XynA} from *Pseudomonas fluorescens* to alanine has no effect on binding (Poole et al., 1993). In contrast, mutation of W68 in CBD_{CenA} of endoglucanase A, also from *C. fimi* (which also corresponds to W72 in CBD_{Cex}), to alanine reduces binding by 30% (Din et al., 1994).

One method to determine the role these exposed tryptophans play in cellulose binding is using chemical modification. In contrast to site-directed mutagenesis, this approach allows features of the *native* protein, such as the thermodynamics or kinetic parameters of ligand binding, to be investigated. Tryptophans in proteins can be oxidized selectively with *N*-bromosuccinimide, allowing a specific analysis of their function. The reaction is rapid and irreversible, converting the indole side chain to an oxindole. Although the size of the modified residue is not significantly greater than that of tryptophan, the two side chains differ in their structural and physical properties, including planarity, aromaticity, and hydrophobicity (Lundblad, 1991). Consistent with this change, there is a loss of tryptophan fluorescence and a decrease in $A_{280\text{nm}}$ as the indole moiety is oxidized to oxindole (Green & Witkop, 1964).

A previous example of the use of chemical modification to study the role of tryptophans in cellulose binding involves endoglucanase II from the fungus *Trichoderma reesei*. EGII comprises a catalytic domain connected to a CBD from family I by a linker. The protein contains 11 tryptophans, 9 in the catalytic domain, and 2 in the CBD. CBDs in family I are about 35 amino acids long, folded into wedge-shaped, three-stranded β -barrels. Four aromatic residues, either tyrosine or tryptophan, are strictly conserved in these CBDs. Three of the aromatic residues are exposed on a hydrophobic face of the wedge (Kraulis et al., 1989). In CBD_{EGII}, one of these exposed aromatic amino acids is a tyrosine, two are tryptophans (Saloheimo et al., 1988). Treatment of EGII with NBS oxidizes three tryptophans, affecting both binding and catalytic activity. The first tryptophan oxidized by NBS is the exposed

tryptophan in CBD_{EGII}; this reduces binding to cellulose by 50%. The second tryptophan to be oxidized is in the catalytic domain (Macarron et al., 1995). Site-directed mutation supports the conclusion that exposed aromatic amino acids of family I CBDs are important in binding to cellulose (Reinikainen et al., 1992). Thus, oxidation of the tryptophans in CBD_{Cex} with NBS could be used to analyze in some detail the interaction of this CBD with cellulose.

The use of fourth-derivative absorbance spectra allows an extremely sensitive measurement of tryptophan oxidation by NBS without interference from residual absorbance at 280 nm (Bray et al., 1994). Additionally, oxidation of the indole ring is readily detectable by NMR spectroscopy, thereby providing a method to identify specifically the tryptophans in a protein that react with the oxidizing reagent. Unfortunately, CBD_{Cex} does not bind tightly soluble cellooligosaccharides, such as cellohexaose, so binding has to be studied with cellulose, which makes kinetic analyses very difficult. Fluorescence spectroscopy offers a convenient method to measure the reaction of NBS with CBD_{Cex} adsorbed to cellulose.

This paper describes the effects of oxidizing the tryptophans in CBD_{Cex} with NBS on the structure and cellulose-binding properties of the polypeptide. In addition to showing that when the surface tryptophans are modified, binding is eliminated, it reveals two unusual properties of the polypeptide: the exposed tryptophans are oxidized preferentially but, unlike most other proteins, NBS oxidizes all of the tryptophans; and binding to cellulose does not prevent oxidation of any of the tryptophans, but it does reduce their sensitivity to the reagent.

Results

Modification of CBD_{Cex} by NBS

Oxidation of the tryptophan residues in CBD_{Cex} was monitored readily by ultraviolet absorbance spectroscopy (Fig. 2). All five tryptophans in the protein were susceptible to chemical modification, with complete oxidation at an NBS:Trp molar ratio of only

Fig. 2. Fourth-derivative spectrum of native CBD_{Cex} (—) in 50 mM sodium acetate, pH 4.0. The Trp-specific trough ($\delta^4 A / \delta \lambda^4$)_{Trp} at 294.5 nm is indicated by the arrow. The fourth-derivative spectrum of CBD_{Cex} fully oxidized by NBS (---), shows that the ($\delta^4 A / \delta \lambda^4$)_{Trp} is eliminated by the modification. Scans were performed at 100 nm min⁻¹, with a fourth-derivative $\delta \lambda$ of 2 nm.

Fig. 3. Effect of oxidation on the binding of CBD_{Cex} to cellulose. Samples of CBD_{Cex} ($8 \mu\text{M}$, equivalent to $40 \mu\text{M}$ tryptophan) in 50 mM sodium acetate, $\text{pH } 4.0$, were treated with increasing amounts of NBS. The number of tryptophans oxidized in the protein and the binding of the oxidized polypeptide to BMCC were assayed as described in the Materials and methods. Inset: Oxidation of the tryptophans in CBD_{Cex} as a function of NBS concentration. CBD_{Cex} ($6 \mu\text{M}$) was treated with the indicated amounts of NBS. The extent of oxidation was calculated as described in Materials and methods.

1.5 (Fig. 3, inset). Complete oxidation of accessible tryptophans in proteins by NBS usually requires 2–4 molar equivalents of this reagent (Lundblad, 1991). Therefore, all of the tryptophans in CBD_{Cex} appear to be very susceptible to oxidation. When present in excess, NBS may also cleave the polypeptide backbone of a protein (Patchornik et al., 1960). However, SDS-PAGE demonstrated that the fully oxidized CBD_{Cex} remained intact (not shown).

Disruption of cellulose binding by NBS oxidation

The binding of CBD_{Cex} to BMCC was reduced 70% by the oxidation of approximately one tryptophan per protein molecule and 90% by the oxidation of two tryptophans (Fig. 3). Complete oxidation of all five indole side chains reduced the binding only a further 5%, possibly because of a low level of residual adherence of the oxidized CBD to the matrix. Therefore, oxidation of approximately two tryptophan side chains is sufficient to abolish the specific binding of CBD_{Cex} to cellulose.

Modification of cellulose-bound CBD_{Cex}

Absorbance spectroscopy cannot be used to monitor modification of CBD_{Cex} by NBS when the protein is bound to insoluble substrates such as BMCC. However, fluorescence spectroscopy, which utilizes incident light at right angles to the sample, can be used in the presence of insoluble substances (Gabel et al., 1971). CBD_{Cex} , when excited at a wavelength of 295 nm , gives a fluorescence maximum at approximately 342 nm , indicative of tryptophans relatively exposed to solvent (Longworth, 1983). Because there are no tyrosine residues in CBD_{Cex} and the fluorescence of phenylalanine is negligible in this region of the spectrum, the observed

signal was an average of the emission of the five tryptophans in CBD_{Cex} , uncomplicated by the presence of other aromatic residues.

In the absence of cellulose, titration of CBD_{Cex} with 1.2 molar excess NBS per tryptophan residue eliminated completely the observed fluorescence at 342 nm (Fig. 4). Adsorption of CBD_{Cex} to BMCC did not prevent oxidation by NBS, but increased the molar equivalent required for complete oxidation under the experimental conditions by 2.4-fold (Fig. 4). Therefore, the tryptophan side chains in CBD_{Cex} are more resistant to oxidation when the polypeptide is adsorbed to cellulose.

Kinetic analysis of CBD_{Cex} oxidation by NBS

Ultraviolet absorbance and fluorescence spectroscopy clearly demonstrate that the tryptophans in CBD_{Cex} are susceptible to oxidation. However, these techniques do not readily distinguish whether, at intermediate levels of oxidation, all five tryptophans are equally oxidized or some tryptophans are completely oxidized and others unperturbed.

To address this question, stopped-flow spectrophotometry was used to determine if the tryptophans in CBD_{Cex} differed in their relative rates of reaction with NBS. At an NBS:tryptophan ratio of 2.0, which is sufficient to completely oxidize all five indole rings, the decrease in $A_{280\text{nm}}$ was biphasic. A rapid phase was completed in less than 1 s and a slower phase lasted for up to 19 s (Fig. 5). The apparent rate constants (k_{app}) for the oxidation of the tryptophans at various concentrations of NBS were calculated from the experimental curves by fitting the data to a single exponential equation. The k_{app} for the reaction decreased as a function of NBS concentration, with the fastest apparent reaction rate observed with a concentration of NBS sufficient to oxidize the equivalent of one tryptophan (Fig. 6). Increasing the NBS concentration above this reduced the rate, but there was no further reduction in rate with concentrations of NBS sufficient to oxidize more than the equivalent of three tryptophans. Based on these measurements, NBS appears to preferentially oxidize three of the tryptophans at low

Fig. 4. Fluorescence of CBD_{Cex} ($3.4 \mu\text{M}$) at 342 nm is plotted against the concentration of NBS added in the absence (●) and presence (○) of 2 mg BMCC/mL . The excitation wavelength was 295 nm . Details are given in Materials and methods.

Fig. 5. Decrease in the absorbance of CBD_{Cex} at 280 nm during reaction with NBS was monitored by stopped-flow spectroscopy. The concentration of CBD_{Cex} was 45.5 μ M, that of NBS 0.45 mM, both in 50 mM sodium acetate buffer, pH 4.0.

reagent concentrations, leaving the two remaining residues, which are oxidized only at higher NBS concentrations, virtually intact.

NMR analysis of the oxidation of CBD_{Cex} by NBS

The chemical shifts of tryptophan side chains are extremely sensitive, site-specific indicators of structural or chemical perturbations of a protein. Therefore, two-dimensional ^1H - ^{15}N correlation spectroscopy was employed to monitor the effects of NBS on the tryptophan residues in uniformly ^{15}N -enriched CBD_{Cex}.

Fig. 6. Apparent rate coefficients k_{app} for the oxidation of CBD_{Cex} as a function of the concentration of NBS. The k_{app} values were calculated by fitting the reaction curve data with a single exponential decay equation using Applied Photophysics curve-fitting software.

The ^1H - ^{15}N HSQC spectrum of CBD_{Cex} at pH 4.0 in 50 mM sodium acetate (Fig. 7A) was virtually identical to that assigned previously at pH 7.0 in water (Xu et al., 1995). This allowed the immediate identification of $^1\text{H}^{\epsilon 1}$ - $^{15}\text{N}^{\epsilon 1}$ resonances of the five indole side chains in the native polypeptide. The intensities of the indole $^1\text{H}^{\epsilon 1}$ - $^{15}\text{N}^{\epsilon 1}$ resonances of the surface tryptophans W17, W54, and W72 diminished as the extent of oxidation was increased, disappearing altogether at an NBS:tryptophan ratio of 0.65 (Fig. 8). Concomitantly, several dispersed, aliased signals appeared in the HSQC due to the resulting oxindolealanine product. The $^1\text{H}^{\epsilon 1}$ indole resonances for the unoxidized tryptophans at these three positions also shifted slightly downfield as the extent of oxidation was increased (Fig. 8). This indicated changes in the local environments of the unoxidized W17, W54, and W72 upon partial oxidation of the neighboring exposed tryptophans in CBD_{Cex}. In contrast, the chemical shifts and intensities of the $^1\text{H}^{\epsilon 1}$ - $^{15}\text{N}^{\epsilon 1}$ resonances from the buried tryptophans W12 and W38 remained unchanged up to an NBS:Trp ratio of 0.65 (Fig. 8). This demonstrates unambiguously that the exposed tryptophans in CBD_{Cex} are oxidized preferentially by NBS, and that oxidation of these three residues does not perturb the overall structure of the protein, as evidenced by the invariant chemical shifts of the two buried tryptophan side chains.

Oxidation of the equivalent of slightly more than three tryptophans caused an overall loss of chemical shift dispersion of CBD_{Cex}, and the appearance of amide ^1H - ^{15}N resonances with random coil chemical shifts (Fig. 7B; Wishart et al., 1991). This is indicative of protein denaturation. At an NBS:Trp ratio of 1.2, all five tryptophans in CBD_{Cex} were oxidized completely, and the backbone and side-chain amides exhibited random coil chemical shifts (not shown). Therefore, the complete oxidation of CBD_{Cex} disrupted its native structure.

Discussion

Oxidation of CBD_{Cex} by NBS

The pattern of CBD_{Cex} modification by NBS is entirely consistent with the structure of the protein (Fig. 1). Tryptophans W17, W54, and W72 lie along one face of the β -barrel and are completely exposed to the solvent, as well as the NBS reagent. The accessible surface areas of the indole rings of these three residues in the mean minimized solution structure of CBD_{Cex} are 100, 147, and 98 $\text{\AA}^2$, respectively (Xu et al., 1995). In contrast, the indole side chains of W12 and W38 are almost entirely buried within the core of the protein, having accessible surface areas of only 7 and 1 $\text{\AA}^2$, respectively.

In general, NBS oxidizes exposed but not buried indole side chains in folded proteins. For example, only 4 of the 8 tryptophans in xylanase A from *Schizophyllum commune* (Bray & Clarke, 1995) and 3 of the 11 tryptophans in EGII from *T. reesei* (Macarron et al., 1995) are oxidized by NBS. Therefore, it is somewhat unusual that all five tryptophans in CBD_{Cex} are susceptible to oxidation.

However, W12 and W38 are not completely sequestered within the interior of CBD_{Cex}, and may still be accessible to NBS, if only transiently, through protein fluctuations. Microcalorimetry reveals that the native state of CBD_{Cex} is not highly stabilized; significant structural fluctuations are likely under the experimental conditions used in this study (C. Haynes, pers. comm.). In addition, oxidation of the exposed tryptophans could destabilize CBD_{Cex}, thereby enhancing the reactivities of the two buried tryptophans. Oxidation of one or both of the buried tryptophans leads to denaturation of the

Fig. 7. ^1H - ^{15}N HSQC spectra of CBD_{Cex} , uniformly labeled with ^{15}N , (A) before and (B) after oxidation by NBS at an NBS:Trp ratio of 0.65. The indole region of the spectrum is indicated as the boxed area in spectrum A.

polypeptide; in the unfolded state, any remaining unreacted indole side chains would be oxidized rapidly by NBS. Chemical modification of aromatic amino acids reduces the stabilities of other carbohydrate-binding proteins, including mushroom lectin (Kawagishi & Mori, 1991), lysozyme (Norton & Allerhand, 1976), an endoglucanase from *Bacillus* (Kawaminami et al., 1994), cardio-

toxin γ from the snake *Naja nigrocollis* (Roumestand et al., 1994), and glucoamylase from *Aspergillus niger* (Svensson et al., 1986).

As shown in Figure 8, the ^1H and ^{15}N chemical shifts of W12 and W38, as well as the main-chain amides of CBD_{Cex} , are unperturbed during oxidation of the three exposed tryptophans. This indicates that there is little significant change in the conformation of the protein when W17, W54, and W72 are converted to oxindolealanine at limiting ratios of NBS to protein (Fig. 7B). It is unlikely that the exposed tryptophans on CBD_{Cex} react differentially with NBS. Oxidation of the equivalent of one tryptophan in glucoamylase from *A. niger* yields subpopulations of molecules in which different tryptophan residues are oxidized partially (Svensson et al., 1986). This appears also to be the case with the exposed tryptophans of CBD_{Cex} . However, as modification of these exposed tryptophans in CBD_{Cex} proceeds, the ^1H and ^{15}N chemical shifts of the indole rings of the unmodified residues at these positions are changed (Fig. 8). This suggests that there are interactions between W17, W54, and W72, or with other residues on the surface of the native CBD, that are affected by the oxidation. It is striking that the progressive modification of the exposed tryptophans appears to cause stepwise shifts in the resonances from the unmodified W17, W54, and W72 indole rings. The reason for this is not readily apparent.

Fig. 8. Preferential oxidation of exposed tryptophans in CBD_{Cex} . Overlap of the indole region of the HSQC spectra (boxed region in Fig. 7A) showing the effects of the oxidation of native ^{15}N -labeled CBD_{Cex} with NBS. Shifts of the indole peaks are in the direction of the arrow, and are of the native CBD, and those at NBS:Trp ratios of 0.13, 0.40, 0.52, and 0.65.

Oxidation of tryptophans affects binding of CBD_{Cex} to cellulose

Oxidation of the equivalent of one tryptophan reduces the binding of CBD_{Cex} to cellulose by 70%, whereas oxidation of the equivalent of two of these groups virtually destroys the ability of the protein to bind to cellulose. Because limiting NBS oxidizes the exposed tryptophans exclusively without perturbing the overall structure of the polypeptide, these residues must play a direct role in binding to cellulose. It appears that the oxidation of any one of W17, W54, and W72 decreases cellulose-binding. This conclusion will be tested by site-directed mutation of each of them.

The direct role of tryptophan residues in cellulose-binding is supported further by three observations. First, the presence of cellulose increases by 2.4-fold the concentration of NBS required to completely oxidize all five tryptophans in CBD_{Cex}. Therefore, the cellulose does appear to make the tryptophans less accessible. Second, the addition of cellobiose perturbs the chemical shifts of the indole rings of W54 and W72 (Xu et al., 1995). Although CBD_{Cex} binds the soluble oligosaccharide very weakly, this provides clear evidence for the interaction of these two exposed tryptophans with cellulose. Third, site-directed mutation of the corresponding tryptophans in other CBDs from family II also disrupts cellulose-binding (Poole et al., 1993; Din et al., 1994).

Currently, CBD_{Cex} is the only member of family II of CBDs for which the three-dimensional structure is known. However, it is certain that all of these related CBDs also adopt β -barrels structures with conserved tryptophans exposed along one face of the molecule. For example, in CBD_{CenA} of endoglucanase A from *C. fimi*, W14 and W68 correspond to W17 and W72, respectively, in CBD_{Cex}. The mutation of W14 or W68, which are predicted to be exposed in CBD_{CenA}, to alanine reduces the K_a values of this protein for binding to cellulose by 50- and 30-fold, respectively (Din et al., 1994). This indicates that the mechanism of cellulose binding by CBD_{Cex} and CBD_{CenA} is similar. In CBD_{XynA} of xylanase A from *Pseudomonas fluorescens* subsp. *cellulosa*, W12, W34, W49, and W66 correspond to W17, W38, W54, and W72, respectively, in CBD_{Cex}. CBD_{XynA} contains a fifth tryptophan, W38, which corresponds to F42 in CBD_{Cex}. The mutation of W12 or W49, which are expected to lie on the surface of the protein, reduces binding by 3- and 10-fold, respectively (Poole et al., 1993). However, the mutation of the surface W66 to alanine has no significant effect on binding, whereas mutation of W34 or W38, which are predicted to be buried in CBD_{XynA}, to alanine eliminates the binding of this CBD to cellulose.

The direct interaction of aromatic residues with carbohydrate rings is a common theme in protein-sugar recognition (Vyas, 1991). Oxidation of tryptophan by NBS changes significantly the physical and chemical properties of the indole side chain, thereby disrupting favorable interactions with the glucopyranose rings of cellulose.

Cellulose-binding by CBD_{Cex} is dynamic

Amino acid side chains in proteins that are involved in substrate binding or catalytic activity can often be protected from chemical modification by the presence of the substrate (Bray & Clarke, 1994, 1995). The binding of CBD_{Cex} to cellulose appears to be irreversible: CBD_{Cex} is not desorbed from cellulose by protracted washing with buffer (Ong et al., 1989, 1991). Surprisingly, although the presence of crystalline cellulose increases the concentration of NBS required for complete oxidation of CBD_{Cex}, it does not prevent modification of any of the five tryptophans. This suggests that the interaction of CBD_{Cex} with cellulose may be dynamic, involving multiple reversible interactions. When bound, exposed tryptophans would be less accessible to NBS. However, modification of any one of them would reduce the affinity of the CBD for cellulose, thereby making the others more accessible to the oxidizing reagent.

Summary

It is clear that the binding of CBDs like CBD_{Cex} to cellulose is a complex process, one of the most striking characteristics of which is its apparent irreversibility. Binding must involve multiple re-

versible interactions between glucose molecules in the cellulose and amino acids in the polypeptide. Although the surface tryptophans are crucial in these interactions, other residues are probably also involved. Site-directed mutants of CBD_{Cex} are being analyzed to evaluate further the roles of particular amino acids in binding.

Materials and methods

Materials

BMCC was prepared as described previously (Gilkes et al., 1992). CF1 cellulose and NBS were from Sigma Chemical Co. (St. Louis, Missouri). SDS, protein molecular weight standards, and 40% acrylamide/bis solution (acrylamide:*N,N'*-methylenebisacrylamide) were from Bio-Rad (Hercules, California). All other chemicals used were of reagent grade, and were purchased from Fisher Scientific (Toronto, Ontario). NBS was recrystallized from water prior to use.

CBD_{Cex} preparation

The vector pTug EO7K3 (Graham et al., 1995) was used to express the gene fragment encoding CBD_{Cex} in *Escherichia coli* strain TOPP5 (Stratagene Inc.). Cultures were grown at 30 °C and pH 7 in M9 minimal medium using a fed-batch procedure (Gilkes et al., 1992). CBD_{Cex} was purified from culture supernatants by affinity chromatography on CF1 cellulose (Ong et al., 1993). It was desalted and concentrated using a Filtron Ultrasette (1-kDa cut-off) tangential flow filtration unit, then applied to a Pharmacia Superose 12 column, pre-equilibrated with 200 mM potassium phosphate buffer, pH 7.0. The same buffer was passed through the column at 0.4 mL/min. Fractions containing protein were pooled and concentrated using an Amicon ultrafiltration unit with a Filtron 1-kDa filter. The purified protein gave a single band of approximately 11 kDa when subjected to SDS-PAGE. Uniformly ¹⁵N-labeled CBD_{Cex} was produced as described previously (Xu et al., 1995).

Analytical methods

Concentrations of CBD_{Cex} were determined spectrophotometrically using $\epsilon_{280} = 2.55 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ (Ong et al., 1993), or by amino acid analysis using an Applied Biosystems 420A/H automated analyzer according to the manufacturer's instructions. The concentrations of the NBS-oxidized derivatives of CBD_{Cex} were determined using $\epsilon_{250} = 6 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ for 3-methoxyindole (Green & Witkop, 1964).

Ultraviolet absorption was measured with a Hitachi U-2000 double-beam spectrophotometer. Fourth-derivative spectra were obtained using the built-in derivative mode ($\delta\lambda = 2 \text{ nm}$). The concentration of tryptophan was determined directly from the fourth-derivative of the parent scan of a protein sample by measuring the depth of a tryptophan-specific trough with a minimum occurring between 292 and 295 nm $\{(\delta^4 A / \delta\lambda^4)_{\text{Trp}}\}$ (Bray et al., 1994). Fluorescence measurements were made with a Perkin-Elmer LS-50 luminescence spectrofluorimeter. Stopped-flow kinetics were measured with an Applied Photophysics Bio-Sequential SX-17MV sequential stopped-flow ASVP spectrofluorimeter, used in the absorbance mode. NMR spectra were obtained with a Varian Unity 500-MHz spectrometer equipped with a pulsed field gradient triple resonance probe, with an actively shielded z gradient coil, as described previously (Xu et al., 1995).

CBD_{Cex} binding to BMCC was assayed using individual samples as described previously (Ong et al., 1993), except that mixtures were incubated for 1 h at 4 °C instead of overnight. Residual binding was calculated from the difference between pairs of samples incubated for the same times with and without added NBS.

Chemical modification of CBD_{Cex}

All reactions with NBS were at ambient temperature. Oxidation of tryptophan to oxindolealanine with NBS was performed routinely by titrating 0.5–0.8 mL of a solution of 6.0–10.0 µM CBD_{Cex} in 50 mM sodium acetate buffer, pH 4.0, with 1–10 µL aliquots of 0.5 mM NBS, made up freshly in the same buffer. Following each addition, the mixture was incubated at 20 °C for 2 min and a parent scan of the sample was acquired from 320 to 240 nm, to monitor the increased absorbance at 250 nm (oxindolealanine) and the decreased absorbance at 280 nm (tryptophan). Samples were also removed after each addition, stored on ice until the end of the experiment, then used to measure adsorption of CBD_{Cex} to cellulose. Parent scans were converted subsequently to fourth-derivative spectra. The titration process was repeated until the ($\delta^4 A/\delta \lambda^4$)_{Trp} at 294.5 nm became zero (Fig. 2) and there were no further changes in the absorbance of the sample at 280 nm. The number of tryptophans oxidized by NBS was calculated by the absorbance method (Spande & Witkop, 1967).

Fluorescence spectroscopy

Fluorescence of CBD_{Cex} was measured in 50 mM sodium acetate buffer, pH 5.0, at a protein concentration giving $A_{280\text{nm}} < 0.1$ (3.4 µM) to minimize inner-filter effects (Hélène et al., 1969). Fluorescence intensities were determined using an excitation wavelength of 295 nm, and then either scanning the sample from 310 to 450 nm, or continuously monitoring emission at 342 nm (excitation and emission slits set at 5 nm). NBS titrations were performed as described above for absorbance measurements. The fluorescence intensity, determined after each addition of oxidant, was corrected for dilution and expressed as a percentage of relative fluorescence intensity of the unmodified protein. NBS titrations of CBD_{Cex} in the presence of BMCC were performed by pre-incubating 3.4 µM CBD_{Cex} with 2 mg BMCC/mL.

Stopped-flow spectroscopy

Kinetic measurements of the reaction of NBS with CBD_{Cex} were made by mixing equal volumes of solutions of 45.5 µM CBD_{Cex} and 0.2–0.5 mM NBS, both in 50 mM sodium acetate buffer, pH 4.0. The dead-time of the instrument was 5 ms, and the sampling time of the reaction was varied from 10 to 50 s. Final measurements were taken using a full scale of 2.0 absorbance units and a sampling time of 20 s. The apparent rates of CBD modification at varying concentrations of NBS were obtained by fitting the data with a single exponential decay equation using Applied Photo-physics curve-fitting software.

NMR spectroscopy

Two-dimensional ¹H-¹⁵N HSQC experiments were run at 30 °C using the sensitivity-enhanced pulsed-field gradient experiment (Kay et al., 1992). A selective water flip-back pulse was incorporated to

ensure minimum perturbation of the water magnetization (Zhang et al., 1994). The protein sample was 0.7 mM uniformly labeled ¹⁵N-CBD_{Cex} dissolved in 50 mM sodium acetate, pH 4.0, 10% (v/v) D₂O. Titration was performed by transferring the protein solution from the NMR tube to a rapidly stirred Pierce Reacti-Vial at 20 °C. Aliquots (10–20 µL) of a 27.5-mM NBS stock solution in the same buffer were added slowly into the vortex of the stirred CBD_{Cex} sample. The protein solution was transferred immediately back to the NMR tube for analysis.

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