Structural Analysis of the Complement Control Protein (CCP) Modules of GABA$_B$ Receptor 1a

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** § $\$§$ The on-line version of this article (available at http://www.jbc.org) contains "Supplemental Results.

The γ-aminobutyric acid type B (GABA$_B$) receptor is a heterodimeric G-protein-coupled receptor. In humans, three splice variants of the GABA$_B$ receptor 1 (R1) subunit differ in having one, both, or neither of two putative complement control protein (CCP) modules at the extracellular N terminus, prior to the GABA-binding domain. The in vivo function of these predicted modules remains to be discovered, but a likely association with extracellular matrix proteins is intriguing. The portion of the GABA$_B$ R1a variant encompassing both of its CCP module-like sequences has been expressed, as have the sequences corresponding to each individual module. Each putative CCP module exhibits the expected pattern of disulfide formation. However, the second module (CCP2) is more compactly folded than the first, and the three-dimensional structure of this more C-terminal module (expressed alone) was solved on the basis of NMR-derived nuclear Overhauser effects. This revealed a strong similarity to previously determined CCP module structures in the regulators of complement activation. The N-terminal module (CCP1) displayed conformational heterogeneity under a wide range of conditions whether expressed alone or together with CCP2. Several lines of evidence indicated the presence of native disorder in CCP1, despite the fact that recombinant CCP1 contributes to binding to the extracellular matrix protein fibulin-2. Thus, we have shown that the two CCP modules of GABA$_B$ R1a have strikingly different structural properties, reflecting their different functions.

The γ-aminobutyric acid (GABA)$_3$ is the principal inhibitory neurotransmitter of the vertebrate central nervous system. It is the ligand for both ionotropic GABA type A receptors and metabotropic GABA type B (GABA$_B$) receptors. GABA$_B$ receptors belong to G-protein-coupled receptor class III, which includes metabotropic glutamate receptors, Ca$^{2+}$-sensing receptors, and some pheromone and taste receptors (1). Agonist and antagonists of GABA$_B$ receptors have been shown to be effective in clinical cases or animal models of nociception, depression, addiction, epilepsy, and cognitive impairment. Selective GABA$_B$ Receptor ligands could also be useful in the treatment of peripheral nervous system disorders (2, 3).

The GABA$_B$ receptor is composed of subunits termed GABA$_B$ R1 and GABA$_B$ R2, both of which are needed for receptor function (4–7). The two subunits share a similar molecular architecture, common to all class III G-protein-coupled receptors, consisting of a large extracellular N-terminal domain encompassing a ligand-binding site, followed by a transmembrane heptahelical domain and an intracellular C-terminal tail. Coupling to G-proteins is mediated by the intracellular loops connecting the transmembrane helices and the C-terminal region.

In class III G-protein-coupled receptors, the extracellular domain of each subunit is proposed to have a dynamic bilobate structure, where the two globular lobes form a "clamshell"-like shape. The current model for the function of these dimers suggests that upon ligand binding, closure of the two lobes in only one of the two domains is sufficient to create a relative change in orientation between the two subunits, which in turn results in the activation of G-proteins (8). In the case of GABA$_B$ receptors, this model is consistent with the observations that only the GABA$_B$ R1 subunit is able to bind GABA$_B$ agonists or antagonists with measurable potency (5, 6), that the GABA$_B$ R2 subunit is important for coupling to G-proteins (9, 10), and

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that the heptahelical region of the GABA<sub>B</sub> R1 subunit also influences coupling efficacy (11). Further evidence that the GABA<sub>B</sub> R1 and GABA<sub>B</sub> R2 subunit extracellular domains are in direct contact is provided by time-resolved fluorescence resonance energy transfer experiments (12).

With regard to the GABA<sub>B</sub> R1 subunit, three splice variants are defined by the presence of one (human GABA<sub>B</sub> R1c), two (human/rat GABA<sub>B</sub> R1a), or neither (human/rat GABA<sub>B</sub> R1b) of the putative complement control protein (CCP) modules at the N terminus, prior to the GABA-binding domain (13, 14). Two other variations of the GABA<sub>B</sub> R1 subunit occur: in the fifth extracellular loop (rat GABA<sub>B</sub> R1c) and at the C terminus (human GABA<sub>B</sub> R1d) (13, 15). Finally, GABA<sub>B</sub> R1e is a soluble truncated version of GABA<sub>B</sub> R1c and differs from GABA<sub>B</sub> R1a in that it has a 62-amino acids deletion that removes the second CCP module-like sequence. The function of GABA<sub>B</sub> R1c has not been reported, but reverse transcription-PCR studies showed up-regulation of this variant in fetal brain (13). Thus, it is the presence of the putative N-terminal CCP modules that is the differentiating factor between the three of the principal variants discovered so far. Significant differences in expression levels within tissues and during development occur between GABA<sub>B</sub> heterodimers containing GABA<sub>B</sub> R1a versus those containing GABA<sub>B</sub> R1b (13). There are, however, no known differences in the pharmacological profiles of the two (17). In this respect, observations that the putative CCP modules of GABA<sub>B</sub> R1a interact with the extracellular matrix are intriguing (17–21).

The 143 residues distinguishing GABA<sub>B</sub> R1a from GABA<sub>B</sub> R1b are thought to form a tandemly arranged pair of CCP modules (22, 23), the only examples of CCP modules suspected to occur in any seven-transmembrane domain receptor. CCP modules are the predominant module type within several soluble and cell-surface regulators of complement activation (24), but another example of a central nervous system protein that contains CCP modules is the 87.6-kDa human equivalent to the mouse SEZ-6 (25, 26). SEZ-6 is a single transmembrane domain mouse protein of unknown function whose expression is enhanced by perfusion of brain slices with convulsant drugs. Although some examples of CCP modules act merely as structural or spacer units in bigger proteins, wherever they occur toward the N terminus of a well studied cell-surface protein, they have been shown to participate in specific protein-protein interactions (22).

The three-dimensional structure of a typical CCP module has a compact hydrophobic core containing conserved residues sandwiched between small antiparallel β-sheets. Four conserved cysteines are disulphide-linked Cys-I–Cys-II and Cys-III–Cys-IV. The sequence of the first putative CCP module of GABA<sub>B</sub> R1a (CCP1) is a less typical example of a CCP module than the second CCP module-like sequence (CCP2) and has an insertion of 12 residues (Arg<sup>43</sup>–Asn<sup>54</sup>; numbering refers to the rat GABA<sub>B</sub> R1a amino acid sequence and includes the signal sequence) that would be expected to be part of the “hypervariable” loop (22). There is also an N-terminal extension of seven residues (Gly<sup>17</sup>–Asn<sup>23</sup>) in CCP1 that is not part of the CCP module consensus. N-Glycosylation sites found in the GABA<sub>B</sub> R1a CCPs are exclusively located in the CCP1 amino acid sequence (at Asn<sup>23</sup> and Asn<sup>68</sup>). We now show that the two CCP modules of GABA<sub>B</sub> R1a have striking structural differences. The first module is not compactly folded, whereas CCP2 is a regular CCP module with particularly high structural similarity to the third module of the decay-accelerating factor (DAF) of complement. Despite being poorly structured, CCP1 exhibits the standard disulfide pattern and is able, like the intact GABA<sub>B</sub> R1a subunit, to bind the extracellular matrix protein fibulin-2.

**EXPERIMENTAL PROCEDURES**

**Construction of Expression Vectors encoding CCP1, CCP2, and CCP12—** DNA fragments encoding rat CCP1 (Gly<sup>17</sup>–Asn<sup>23</sup>), CCP2 (Val<sup>30</sup>–Asn<sup>68</sup>), and CCP12 (Gly<sup>17</sup>–Asn<sup>130</sup>) were amplified by PCR using a DNA template prepared by Dr. Edward Hawrot (Brown University, Providence, RI). The sense primer used to amplify the CCP1 and CCP12 DNA fragments was designed to include the first seven residues of the mature rat GABA<sub>B</sub> R1a gene product so that the first residue of the recombinant protein corresponds to Gly<sup>17</sup>, which immediately follows the putative signal peptide sequence. The sense primer for the CCP2 DNA fragment was designed to include the three “linker” residues (Val<sup>30</sup>–Asn<sup>68</sup>) between the fourth consensus Cys of CCP1 and the first consensus Cys of CCP2. The antisense primer used to amplify CCP1 and CCP12 DNA fragments was designed to include the three residues following the fourth consensus Cys of CCP2; the antisense primer used to amplify CCP1 was designed to include Val<sup>30</sup>–Asn<sup>68</sup>. All sense primers incorporated an EcoRI restriction site, and all antisense primers incorporated a NotI restriction site as well as two stop codons. Each resulting DNA fragment was digested with EcoRI and NotI and inserted into the same sites of the pGIZcZA yeast expression vector (Invitrogen, Paisley, UK). All plasmids were sequenced to confirm the desired DNA sequence and the correct reading frame. DNA mid-preparations of the expression constructs were prepared using the Wizard Plus midi-preparation DNA purification kit (Promega, Southampton, UK). For each CCP construct, 10 μg of SacI-linearized plasmid were electroporated into E. coli (Invitrogen’s strain KM71H/Mut<sup>3</sup>) according to the recommendations of Invitrogen. Zeocin-resistant colonies were then tested for protein expression; colonies expressing the most recombinant protein were picked for large-scale protein preparations.

**Expression and Isotope Labeling—** For each protein construct, a single colony was used to inoculate a starter culture (10 ml) of buffered minimal glycerol (BMG; 100 mM potassium phosphate (pH 6 or 3), 0.2% (w/v) yeast nitrogen base (YNB); with or without amino acids), 1% (w/v) glycerol, and 0.00004% (w/v) biotin). After 2 days of incubation at 30 °C (250 rpm), this starter culture was diluted to 1 liter with BMG; equal portions were transferred into four 1-liter baffled flasks; and the flasks were shaken at 30 °C for an additional 2 days (Δ<sub>600</sub>~13–15). Cells were then pelleted at 1500 × g for 5 min at room temperature and transferred to 200 ml of buffered minimal methanol medium (100 mM potassium phosphate (pH 6 or 3), 1.34% (w/v) YNB (with NH<sub>4</sub>SO<sub>4</sub> and without amino acids), 1% (w/v) glycerol, and 0.00004% (w/v) biotin). For 15N/YNB isotope labeling, buffered minimal dextrose medium (100 mM potassium phosphate (pH 6 or 3), 0.5% (w/v) malate, and 0.00004% (w/v) biotin) for induction. Inductions were performed over 5 days at 30 °C. Cells were resuspended in fresh induction medium daily, and supernatants were stored at −20 °C with protease inhibitors (1 mg phenylmethylsulfonyl fluoride and 5 mg EDTA) while awaiting purification.

For 15N isotope labeling, cultures were grown in modified BMG in which 1.34% (w/v) YNB was replaced with 0.34% (w/v) YNB (without NH<sub>4</sub>SO<sub>4</sub> and without amino acids) supplemented with 0.2% (w/v) (15NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (15N/YNB). For 13C/15N isotope labeling, a 13C/15N buffered minimal dextrose medium (100 mM potassium phosphate (pH 6 or 3), 0.5% (w/v) (13C)malate, and 0.00004% (w/v) biotin) was used instead of BMG. Cells were then harvested by centrifugation as described above and resuspended in 100 mM potassium phosphate (pH 3), 13C/15N YNB, 0.1% (w/v) (13C)glucose, and 0.00004% (w/v) biotin) for 2 h prior to induction. This stage allows a smooth transition between glucose and methanol as the sole carbon source for the cells and was found to reduce cell death. Induction for both 15N and 13C/15N isotope labeling was carried out using buffered minimal methanol medium containing 13C/15N YNB instead of 14N/YNB. For 13C/15N isotope labeling, buffered minimal methanol medium was prepared with (15C)malate. All iso¬topically labeled compounds were purchased from Cambridge Isotope Ltd. (Cambridge, MA).

**Purification—** Supernatants were filtered through a 0.2-μm filter and concentrated 50-fold using a combination of a preparative scale spiral wound filter module (Millipore, Watford, UK) linked to a peristaltic pump and an N<sub>2</sub>-pressurized stirred cell (Millipore) at 4 °C. The concentrated proteins were purified by cation exchange chromatography (Mono S HR 5/5, Amersham Biosciences, Little Chalfont, UK) with a 0–1 M NaCl gradient over 25 column volumes; CCP1 and CCP12 were buffered in 12.5 mM sodium acetate (pH 5.3), and CCP2 was buffered in 50 mM sodium acetate (pH 4.6). Following this initial cation exchange step, CCP1 and CCP12 were deglycosylated with endoglycosidase H (3000 units/mg of recombinant protein) for 6–8 h at 37 °C. Endoglyco-
sidase H$_2$ was removed by reloading the cleavage mixture onto the Mono S column; traces of glycosylated material were removed by concanava-
ilin-A-Sepharose chromatography (Amersham Biosciences). All recom-
binant fragments were further purified by reverse-phase chromatog-
raphy (RP2 column, Applied Biosystems, Warrington, UK) with a 10–
60% acetonitrile and 0.1% (v/v) trifluoroacetic acid gradient over 34 column volumes. Protein yields were on the order of 5 mg/preparation. The N-terminal sequence of each protein construct was confirmed by amino acid sequencing (Dr. A. Cronshaw, University of Edinburgh, Edinburgh, UK). Protein concentrations were calculated using absorb-

**Mass Spectrometry—** All GABA$_B$R1a CCP fragments were examined by mass spectrometry using positive electrospay ionization on a Mic-
romass Platform-II instrument. The number of disulfide bonds present in
CCP1 and CCP12 was deduced from a series of alkylation/reduction/expe-
riments using dithiothreitol (DTT) and neutralized iodoacetate treatment (27), followed by a combination of reverse-phase chromatog-
raphy and electrospray ionization mass spectrometry. The disulfide bond pattern in a “truncated” version of CCP1 (lacking the seven-
residue N-terminal extension) was analyzed by trypsin digestion and sequencing of the resulting fragments on a Micromass hybrid quadru-
pole time-of-flight mass spectrometer.

**Fluorescence Measurements (near-UV)—** Fluorescence spectra were corrected for the spectral response of the instrument and normalized to the excitation intensity at 280 nm. ANS fluorescence was excited at 380 nm and recorded between 400 and 600 nm. ANS fluorescence was used to monitor the binding of CCP1 to the GABA$_B$R1a CCP fragments.

**Differential Scanning Calorimetry (DSC)—** Calorimetric measure-
ments were carried out in a VP-DSC differential scanning microcal-
orimeter (MicroCal, Northampton, UK) at the Microcalorimetry Facility of the University of Glasgow by Prof. Alan Cooper. The cell volume was 0.5 ml; the heating rate was 1 °C/min; and the excess pressure was kept at 25 p.s.i. All protein fragments were used at a concentration of 40 μM in 20 mM sodium phosphate (pH 7.5). The molar heat capacity of each protein was estimated by comparison with duplicate samples contain-
ing identical buffer from which the protein had been omitted. The partial molar heat capacities and melting curves were analyzed using standard procedures (33).

**1-Anilinonaphthalene-8-sulfonic Acid (ANS) Fluorescence Measure-
ments—** Fluorescence emission spectra were recorded with a Fluoro-
max-3 spectrometer (Jobin-Yvon Ltd., Middlesex, UK). Protein samples (3.6 μM) in 20 mM sodium phosphate (pH 7.5) were mixed with 20 μM ANS (Molecular Probes Europe, Leiden, The Netherlands) prepared in the same buffer and left to equilibrate for 20 min at room temperature. Excitation was at 370 nm, and emission was recorded between 400 and 700 nm using 1.50-nm band-pass excitation and emission slits. Mea-
surements were performed at 20 °C. The fluorescence signal obtained from the buffer alone with 20 μM ANS was subtracted. Measurements were confirmed with samples incubated for longer periods of time (2–3 h) at room temperature or at physiological salt concentrations (phosphate-buffered saline).

**NMR Spectroscopy and Analysis—** All 1H-15N heteronuclear single quantum coherence (HSQC) spectra were recorded on a Varian INOVA

spector at 600 MHz in 20 mM 6°-sodium acetate (pH 4.0) (90% D$_2$O) at 6°C. D$_2$O (5% v/v) was used as a deuterium lock. The HSQC spectrum of CCP1 in H$_2$O was acquired with 1000 Hz in the 1H dimension and 2000 Hz in the 15N dimension. The spectral widths were 5000 Hz in the 1H dimension and 2000 Hz in the 15N dimension for all spectra. The CCP1 spectrum was recorded with 64 scans and 512 increments in the 15N dimension; the CCP2 spectrum was recorded with 8 scans and 128 increments in the 1H dimension; and the CCP12 spectrum was recorded with 16 scans and 96 increments in the 15N dimension. After processing, data matrices were 1024 points in 1H and 57,147 points in 15N dimensions. The number of scans was 12 and 36 (near-UV) and seven scans (near-UV) and was corrected by subtraction of a spec-
trum obtained for a solution lacking the protein but otherwise identical.

**Circular Dichroism Measurements—** All GABA$_B$R1a CCP fragments were examined using the Jasco Model 810 spectropolarimeter. Measurements were recorded at 20 °C in 20 mM sodium phosphate (pH 7.5). The protein concentra-
tions used were 36 and 12 μM in far- and near-UV experiments, respec-
tively. Each CD spectrum represents the average of five scans (far-UV) or seven scans (near-UV) with a 2°C scan increment. Spectra were recorded at 25°C using 10 μM protein in the different protein fragments to achieve a maximum signal-to-noise ratio.

**Fluorescence Microscopy—** GABA$_B$ R1a CCP fragments were examined in living fibroblasts expressing GABAB R1a or GABAB R1b with GABAB R2 subunits. Expression was confirmed by immunocytochemical staining using anti-GABA$_B$R1a or anti-GABA$_B$R1b antibodies and Texas Red-conjugated goat anti-mouse IgG antibodies. Images were collected using a Leica TCS SP5 microscope equipped with a 63× oil-immersion objective. The images were processed using ImageJ software (National Institutes of Health, Bethesda, MD).
Structure of GABA<sub>B</sub> R1a CCP Modules

256 points in 15-N. For each spectrum, a sine-bell square window function (with a shift of 90°) was applied in both dimensions. Unlabeled, 1<sup>5</sup>N-labeled, and 13C/15N-labeled CCP2 samples at a concentration of ~1 mC were prepared in 20 mM sodium acetate (pH 4.0) (90% H<sub>2</sub>O and 10% D<sub>2</sub>O). Spectra were collected at 600 MHz (37 °C) except where stated. For resonance assignments, 1H-15N HSQC (34, 35) and 1H-13C HSQC (36) spectra were used along with the following three-dimensional heteronuclear experiments: CBCA(CO)NH and HNCA/CB (37), HBBH/CA(NH) (38), HBHAN (39), (H/C/O/C/NH-total correlation spectroscopy (TOCSY) and H/C/O/CA/HC(N)/OS) (40), HCC(C/CD)NH and (H/C/O/C/ND)HH(D) and (H/C/O/C/CD)CE(HE) (42), 15N- and 13C- (at 800 MHz) edited NOESY experiments were collected with mixing times of 146 and 100 ms, respectively.

Hydrogen bond donors were identified from relatively slowly exchanging (>20 min) amides in a series of H<sub>2</sub>0/D<sub>2</sub>O exchange 1H,15N HSQC spectra, and proton acceptors were inferred from supporting nuclear Overhauser effect (NOE) data. A semiconstant time heteronuclear multiple/single quantum coherence was used to derive <i>J</i><sub>NNH</sub> values (44). NMR data were processed within AZARA (available at www.bio.cam.ac.uk/azara) making use of maximal entropy methods to process the indirectly detected dimensions in three-dimensional experiments; spectra were viewed and assigned within ANISIG (45).

For 1H <i>T</i><sub>1</sub> measurements, delays of 6.75, 12.2, 328.3, 655.3, and 873.3 ms were used; for 15N <i>T</i><sub>1</sub> measurements, delays of 17.02, 33.02, 65.02, 145.02, 161.02, and 177.02 ms were used (46). For each residue, a single exponential decay was fitted to the extracted peak heights and the correlation time (<i>r</i>) was calculated from the inverse of the proton relaxation rate (<i>T</i><sub>1</sub>). NOEs were calculated for the region of the cross-peaks in the reference spectrum to those recorded in the spectrum in which the 1H signals were saturated (47).

Structure Calculation and Comparisons—A second backbone 15N-1H resonance was observed for each of 27 residues in the 1H,15N HSQC spectrum of CCP2, most of which were found in the region spanning Val<sup>111</sup> to Phe<sup>120</sup>. The second set of resonances was assigned to a minor form of CCP2, in which Pro<sup>119</sup> is in the ambiguous NOE list to remove assignment possibilities that contributed two (for the lowest NOE-derived energies, no violations of distance restraints discarded, and structures were selected on the basis of having the lowest NOE-derived energies, no violations of distance restraints <0.5 Å, and no violations of coupling constants >1 Hz.

Combination of extension (51) was used to compare all 25 solved CCP modules individually against the cis- and trans-forms of the GABA<sub>B</sub> R1a CCP2 module. Multiprot (52) was used to calculate the multiple structure superposition of all solved CCP modules (including both GABA<sub>B</sub> R1a CCP2 forms).

RESULTS

Expression and Purification—<i>P. pastoris</i> is an appropriate organism for expression of CCP modules because it possesses the ability to promote disulfide bond formation and glycosylation (53, 54). In this study, <i>P. pastoris</i> was used to express the putative CCP modules of GABA<sub>B</sub> R1a as a module pair (CCP12) and as single modules (CCP1 and CCP2). All three protein fragments were secreted at useful levels (8–10 mg of protein/liter of BMGM) when the recombinant organisms were grown in shaking flasks. Lower induction temperature (25 °C instead of 30 °C as generally used for cultivating <i>P. pastoris</i>), low pH (pH 3), and cycles of daily harvests, followed by cell resuspension in fresh induction medium, helped to maximize yield. (Note that samples from <i>P. pastoris</i> cultured at pH 3 were identical from a biological standpoint to protein produced by growth at pH 6 (data not shown).)

All fragments were purified by cation exchange chromatography, followed by reverse-phase chromatography. Note that alternative methods of purification, not incorporating a reverse-phase step, produced proteins with identical spectra (data not shown). Consensus N-glycosylation sites occur at Asn<sup>23</sup> and Asn<sup>83</sup>; and in this work, CCP1 and CCP12 were treated with endoglycosidase H. Following purification, each product showed one band upon SDS-PAGE (Fig. 1). Their identities were confirmed by N-terminal sequencing, which showed the presence of EF (from the DNA cloning strategy) or EAEEF (the EA is left over from cleavage of the yeast α-factor signal sequence by Ste13) prior to the native sequence.

Mass Spectrometry—Electrospray ionization mass spectrometry of the GABA<sub>B</sub> CCP modules expressed in <i>P. pastoris</i> confirmed that their molecular masses match those expected if all cysteines are involved in disulfide formation (see “Supplemental Results”). In addition to a major species bearing one residual GlcNAc unit, minor components with no or two residual GlcNAc units were observed for CCP1 and CCP12. Oxidation of Met<sup>180</sup> was also observed in most but not all CCP1 and CCP12 preparations and had no significant effect as judged by NMR spectroscopy. Tryptsin digestion and quadrupole-time-of-flight mass spectrometry confirmed the expected CCP module pattern of disulfide formation in CCP1.

Binding Experiments—Preliminary experiments carried out using the yeast two-hybrid system with a human brain cDNA library as prey and DNA fragments encoding the CCP modules of human GABA<sub>B</sub> receptors as bait indicated that CCP1 interacts with the C-terminal domain of fibulin-2. To confirm that the GABA<sub>B</sub> receptor CCP modules interact with fibulin-2, TrxF-F2C was immobilized on Sepharose and used to affinity isolate the receptor from solubilized rat synaptic plasma membranes. The results shown in Fig. 2b indicate that both GABA<sub>B</sub> R1a and GABA<sub>B</sub> R1b were isolated from the solubilized synaptic membranes, but that the GABA<sub>B</sub> R1a subunit was substantially enriched in the isolate compared with the GABA<sub>B</sub> R1b subunit. To determine which of the GABA<sub>B</sub> R1 isoforms interacts with F2C, a similar affinity isolation was performed using lysates of cells permanently expressing the GABAB R1a or GABAB R1b subunit. The GABA<sub>B</sub> R1a subunit was selectively isolated by incubation with the Trx-F2C fusion protein, whereas the GABA<sub>B</sub> R1b subunit appeared to be nonspecifically isolated under these conditions (Fig. 2c, Con lanes). In the case of the GABA<sub>B</sub> R1a subunit, both fully and non-glycosylated forms of the receptor were isolated, whereas with the GABA<sub>B</sub> R1b subunit, only the non-glycosylated intracellular pool of the protein was pulled down in both the specific and nonspecific incubations. There appeared to be a larger intracellular pool of the unglycosylated GABA<sub>B</sub> R1b subunit in the cell lysates (Fig. 2c, Lys lanes, compare the lower bands), and some of this material could be poorly folded and consequently give rise to nonspecific interactions with the affinity supports. Consistently, however, the GABA<sub>B</sub> R1a subunit was pulled down specifically in these experiments, suggesting that one or both of the CCP modules are important for the interaction with fibulin-2.

To confirm that the GABA<sub>B</sub> R1a CCP modules, as expressed in <i>P. pastoris</i>, retain the ability of the native receptor to bind fibulin-2, CCP12 was immobilized on Sepharose and then incubated with medium from cells secreting fibulin-2. Under
these conditions, it was clear that fibulin-2 was indeed enriched in the eluates from immobilized CCP12-Sepharose, but not in those from control GST-Sepharose (Fig. 2d, upper panel). In all of these experiments, even when the C-terminally degraded form of fibulin-2 was present, the only form that was isolated by CCP12-Sepharose was full-length fibulin-2. This suggests that the C-terminal domain of fibulin-2 is necessary for the interaction. To determine which of the two CCP modules interacts with fibulin-2, the individual modules were immobilized on Sepharose. As shown in Fig. 2d (lower panel), CCP1 (but not CCP2) pulled down full-length fibulin-2. Thus, by affinity isolation of the native or recombinantly expressed GABAB R1a-GABAB R2 heterodimer with fibulin-2 and by isolation of fibulin-2 with the CCP modules expressed in P. pastoris, we have confirmed results based on yeast two-hybrid studies showing that these modules can interact with fibulin-2.

NMR Spectroscopy—The double module fragment (CCP12) was investigated by NMR spectroscopy prior to expression of the individual modules. Its 1H-15N HSQC spectrum contains an appropriate number of cross-peaks (Fig. 3a), but a proportion of these have relatively low intensity. Furthermore, a lack of transfer in TOCSY-type experiments was apparent (data not shown). Conformational heterogeneity was inferred from inspection of the region of the 1H-15N HSQC spectrum where three tryptophan NH cross-peaks would be expected (∼125–130 ppm 15N and ∼10–10.5 ppm 1H). This inference was subsequently confirmed by analysis of individual modules (Fig. 3, b and c).

The 1H-15N HSQC spectrum of CCP1 is characterized by a mixture of sharp and broad resonances. Many of these signals have poor 1H dispersion, implying that there is a substantial part of CCP1 that is not compactly structured. On the other hand, the presence of a number of dispersed non-glycine resonances in its 1H-15N HSQC spectrum indicates some structure within CCP1. The number of CCP1 cross-peaks and their dispersion in 1H-15N HSQC experiments showed no improvement over a wide range of pH values and under other conditions (including near-physiological conditions); pH 4 was chosen for most of the spectra to reduce solvent exchange of HN resonances. Neither the glycosylation state of CCP1, nor the absence (in the truncated version) of the N-terminal extension of seven residues (Gly17–Asn23) prior to the first consensus Cys, resulted in any significant improvement in the quality of the NMR spectra. The three-dimensional 15N-edited NOESY spectra of CCP1 showed similarly poor overall dispersion, whereas even cross-peaks that are dispersed in the 1H-15N HSQC spectra showed few inter-residue NOEs. Only a small number of NOEs in the NH-NH region were present, and no NOEs could be observed between the high field-shifted methyl groups and HN resonances.

In contrast to the 1H-15N HSQC spectrum of CCP1, the CCP2 spectrum is characteristic of a well folded protein domain. The presence of some doubled cross-peaks is a consequence of cis/trans-isomerization of an X-Pro bond (see below). Standard sets of NMR experiments were collected on a 13C/15N-labeled sample of CCP2, analyzed, and used as a basis for three-dimensional structure calculations (below).

When 1H-15N HSQC spectra (Fig. 3, d and e) and three-dimensional 15N-edited NOESY spectra (data not shown) recorded on CCP1, CCP2, and CCP12 were overlaid, they showed that the spectra of the module pair are simply additions, with only subtle differences, of the individual module spectra. For example, in regions where tryptophan, glycine, and serine/threonine peaks occur often (∼108–113 ppm 15N and ∼8.3–8.7 ppm 1H), most of the resolved spin systems observed in the CCP1 spectra are clearly identifiable in the CCP12 fragment, with no significant change in NOE patterns or chemical shifts. Likewise, most cross-peaks attributable to CCP2 are present in the CCP12 spectra. Few amide protons could be detected in the 1H-15N HSQC spectrum of CCP12 h after redissolving freeze-dried material in D2O at 25 °C (data not shown). All of these were attributable to CCP2 and none to CCP1. Glycosylation of CCP12 did not significantly affect spectral quality (data not shown).
Differential Scanning Calorimetry—The thermal unfolding of the GABAB fragments was studied to probe their thermodynamic stability and tertiary structure. The DSC data for CCP12 and CCP2 (Fig. 4a) are consistent with a single endothermic transition in each case, with very similar midpoints (64.72 ± 0.03 and 66.59 ± 0.04 °C). In both cases, there was a nearly perfect agreement between calorimetric and van’t Hoff enthalpies, which indicates that unfolding can be adequately approximated by a two-state mechanism and that no intermediates are present at equilibrium. No sign of visible precipitation was observed after the recordings. In contrast, the CCP1 DSC profile (Fig. 4b) has no heat absorption peak over the temperature range employed (22–100 °C), although a weak negative transition can be observed at ~52 °C prior to a substantial decline in heat capacity above 60 °C. The latter is probably due to the irreversible exothermic aggregation of the protein, but no evidence of precipitation was seen after the recordings. This result strongly suggests that recombinant CCP1 has little rigid tertiary structure, in contrast to CCP2. Moreover, the similarity of the calorimetric profiles obtained for CCP2 and CCP12 points to CCP1 having a lack of stable tertiary structure in the context of the module pair.

Circular Dichroism Spectroscopy—To complement NMR and DSC studies, far- and near-UV CD spectra were recorded on individual modules. Far-UV CD spectra of proteins are used to estimate their secondary structure content, whereas near-UV CD spectra are sensitive to the environments of aromatic side chains and have contributions from disulfide bonds. It has been previously reported that the far-UV CD spectra of compactly folded CCP modules are characterized by an unusual positive ellipticity in the 220–240 nm region (55); furthermore, it has
been suggested that this characteristic could arise from the presence of tryptophan residues in β-strands (55). The near-UV CD spectra of CCP modules are dominated by a large negative band centered at 280 nm (55).

CCP1 showed a well defined positive ellipticity in the far-UV region centered at 228 nm (Fig. 5a), which was noteworthy in view of the low extent of folding implied by NMR spectroscopy and the lack of thermal stability inferred from the DSC studies. Moreover, CCP1 has a negative band centered at 280 nm (Fig. 5b). A positive ellipticity in the far-UV region (centered at 237 nm) and a very large negative band centered at 280 nm in the near-UV region were observed for CCP2 (Fig. 5, c and d), consistent with its compactly folded nature as determined by NMR and DSC.

CCP modules have two disulfide bonds and a conserved tryptophan that is found within the hydrophobic core of the module, close to the Cys-I–Cys-III disulfide bond. It is therefore possible that the negative ellipticity at 280 nm has contributions from disulfide bonds as well as contributions from aromatic amino acid side chains packed within the core. Either possibility is consistent with the effects of 10 mM DTT, which abolished the negative ellipticity in CCP1 (Fig. 5b). On the other hand, in the case of CCP2, 10 mM DTT diminished the 280 nm signal, but 20 mM DTT was required to abolish it completely (Fig. 5d). This result suggests that the CCP1 disulfide bonds are more accessible to DTT compared with CCP2. Whether the loss of signal in these cases is due to thermal melting of the hydrophobic cores or arises directly from reduction of the disulfide linkages (or a combination of the two) remains unknown.

Upon reduction with 10 mM DTT, the CCP1 far-UV positive ellipticity was abolished to a degree equivalent to that of the denatured (6 M GdnHCl) protein (Fig. 5a). On the other hand, CCP2 retained almost all its far-UV positive ellipticity at 20 mM DTT (Fig. 5d). Disulfide bond contributions to CD spectra are normally seen in the near-UV region, but they can also contribute to the intensity at 225 nm, depending on their dihedral angle (56). The fact that 20 mM DTT (which, based on the evidence of the near-UV spectra, caused reduction of the disulfides in both CCP1 and CCP2) did not abolish the ellipticity of CCP2 at 237 nm implies that, in this case, the signal does not arise from the disulfides. Furthermore, this result indicates that a significant degree of secondary structure remains, even in the absence of disulfide bonds. On the other hand, in the case
of CCP1, no detectable secondary structure remains once the disulfides are reduced.

The relative stability of each module was further investigated by recording far-UV CD spectra as a function of GdnHCl (Fig. 6a). CCP1 showed an almost complete loss of signal above 3.5 M GdnHCl, with a midpoint value of 1.8 M GdnHCl. This is a notably low value for a CCP module and implies that the secondary structural elements that give rise to this negative ellipticity are only marginally stable. By contrast, CCP2 showed little loss of secondary structure below 3 M GdnHCl and a total loss only at 7 M GdnHCl, with a midpoint value of between 5 and 5.5 M GdnHCl. CCP2 is therefore a particularly stable example of a CCP module, with a midpoint value higher than any of the previously reported CCP unfolding transitions. For example, the 16th CCP module of complement receptor type 1 has a midpoint of 4–4.5 M GdnHCl (55).

**ANS Fluorescence Experiments**—A well established and sensitive test to probe partial folding in globular proteins is the binding of the fluorophore ANS. Binding of a protein to ANS induces an increase in the fluorescence of ANS at 470 nm upon excitation at 370 nm. ANS binds to solvent-accessible hydrophobic regions in proteins. Although ANS has been shown to bind to surface-exposed patches of non-polar groups in compactly folded proteins, binding to a partially folded state is much stronger in general compared with the native or fully denatured state (57). ANS was found to bind only weakly to CCP2 (Fig. 6b). This weak binding is attributable to the presence of a hydrophobic patch seen in the NMR-derived structure (see Fig. 9). By contrast, ANS bound strongly to CCP1 (Fig. 6b). This is evidence of the presence of a substantial number of solvent-accessible non-polar groups in CCP1 and implies strongly that CCP1 is not compactly folded.

The double module (CCP12) at the same concentrations as CCP1 and CCP2 in the experiments described above exhibited a substantially higher degree of ANS binding compared with CCP2 alone. This is consistent with the failure of CCP2 to induce a compactly folded conformation of CCP1 in the double module. On the other hand, ANS binding was lower than that observed for CCP1 alone. This suggests that there is some degree of stabilization of CCP1 in the presence of CCP2.

**Three-dimensional Structure of CCP2**—Two ensembles each of 24 three-dimensional structures were calculated on the basis of experimental data: for a major form of CCP2 with the trans-configuration at the Leu$^{118}$-Pro$^{119}$ peptide bond and for a similar (backbone root mean square deviation (r.m.s.d.) = 1.26 Å over all 61 residues) minor form with the cis-configuration at the Leu$^{118}$-Pro$^{119}$ bond (Fig. 7, a and b). Structures representing the trans-form converge slightly better (Table I), reflecting the greater number of inter-residue NOEs available for the calculation. Taken together, the number of experimental restraints (an average of 10 unique and unambiguous inter-residue NOEs/residue in the trans-form), the low number of violations exhibited by the final structures, the good convergence, and the Ramachandran plot (91% of residues in the most “favored” or “additional allowed” regions) indicate that a relatively good quality three-dimensional structure has been obtained for trans-CCP2. This is the first atomic resolution structural data for any part of the GABA$_B$ receptor. The cis- and trans-forms of the structure differ significantly only in the vicinity of the cis-trans-peptide bond (Fig. 7c). Because the cis-form is less populated in the NMR sample, the description below concentrates on the trans-form.

CCP2 has a three-dimensional structure (Fig. 7c) that resembles that of other CCP modules (24). Extended segments of polypeptide, some regions of which may be classified as $\beta$-strands, are connected by loops and turns. The extended segments run antiparallel to each other and form five staves of a barrel-like structure with N and C termini at opposite ends. Up to eight stretches of $\beta$-strand (strands A–H) occur in CCP modules (58), but in the case of trans-CCP2, only strands B and D–H (accounting for 23 residues in total) feature consistently among the 24 members of the ensemble of NMR-derived CCP2 structures. Residues corresponding to strands A and C do not satisfy the criteria of Kabsch and Sander (59) for $\beta$-strands in most cases. With six residues, strand D is the longest $\beta$-strand and forms part of a four-stranded antiparallel $\beta$-sheet with strands B and F (on either side of strand D) and strand G (on the other side of strand F); strands E and H form a separate, small two-stranded antiparallel $\beta$-sheet.

As in several other known examples of CCP modules, within the fourth stave of the barrel, a looped out section occurs between strands E and F (Fig. 7c). In most CCP modules, there is a region of high sequence and structural variation in the second stave, after strand B, termed the hypervariable loop or region (24). This frequently forms a lateral projection and has often been suggested as a likely interaction site. In CCP2, the hypervariable region does not project laterally; instead, it and...
the region corresponding to strand C in the classical CCP module structure form a prominent longitudinal projection. Among the family of 25 solved structures of CCP modules, this projection is unique. The sequence here is LPAL (where the Leu118-Pro119 peptide bond is the source of the cis- and trans-forms of CCP2). In the trans-form, there is little evidence that this loop undergoes mobility on the nanosecond-to-picosecond time scale since heteronuclear NOEs in this region are not noticeably lower than average (Fig. 8a). On the other hand, in the minor cis-form, residues 117 and 118 do have lowered $^{1}H$-$^{15}N$ NOEs consistent with mobility on this rapid time scale. In both forms, several residues in the loop have shorter $T_2$ values and elevated $T_1/T_2$ ratios, suggestive of motion on the millisecond-to-microsecond time scale (Fig. 8b and c). This hypervariable loop lies close to the N terminus of the CCP2 module, and therefore, in the intact receptor, it might be expected to interact with CCP1 and may have different dynamic properties.

In terms of overall structure, trans-CCP2 is most similar (Fig. 7d) to the third module of DAF (r.m.s.d. = 1.49 Å over 52 residues), the second CCP module of β₂-glycoprotein I (r.m.s.d. = 1.57 Å over 57 residues), and the first module of complement receptor type 2 (r.m.s.d. = 1.60 Å over 53 residues). It is least similar to the fifth module of factor H (r.m.s.d. = 3.86 Å over 57 residues). A very unusual feature of the CCP2 sequence is the lack of proline residues in the stretch following the first of the consensus cysteines. This is unique among CCP modules with solved three-dimensional structures, and the average number of proline residues here is two. This segment of CCP2 has a helical appearance (a short helical turn appears in 37.5% of the structure ensemble, including the closest-to-mean structure, with hydrogen bonds from the Ser101 oxygen to Tyr103 HN, the Lys101 oxygen to Leu104 HN, and the Ser102 oxygen to Thr105 HN), but nonetheless traces a similar overall path to the equivalent segments of conventional CCP modules. Compared with the third module of DAF, the second CCP module of β₂-glycoprotein I, and the first module of complement receptor type 2, CCP2 has a longer hypervariable region, which is accommodated in the unique longitudinal projection described above. Compared with the third module of DAF, CCP2 lacks an insertion in the EF loop. Both of these features are near the N terminus. Thus, when CCP2 is overlaid with its closest structural relative, the third module of DAF (Fig. 7e), the two structures are seen to be very similar indeed at their C-terminal ends, but diverge at their N-terminal ends.

CCP2 does not possess any outstanding electrostatic features (Fig. 9a) apart from a negatively charged patch close to its C terminus. The same face of CCP2 also carries several exposed hydrophobic side chains, including Phe112 and Leu113, which are non-conserved (Fig. 9b), whereas the opposite face lacks any notable lipophilic characteristics.
Preparation of CCP Modules from GABAB R1a—The work presented here confirms that CCP1, in both the native and recombinant forms, makes the major contribution to the interaction with fibulin-2. The functional relevance of CCP1 interaction with fibulin-2 is still unknown. Fibulins are present in the central nervous system and are known to have roles in development (61). The GABAB receptor and an extracellular matrix protein. That interaction was shown using pull down-type assays for the first time, thus confirming an intriguing interaction between the GABAB receptor and an extracellular matrix protein. That the CCP12 fragment expressed in P. pastoris could be used in affinity purification of fibulin-2 is also highly significant because this confirms that the material expressed in yeast retains one of the functional properties of the intact receptor. The previously reported yeast two-hybrid experiments were negative for CCP2 binding to fibulin-2 (18, 19). The results presented here confirm that CCP1, in both the native and recombinant forms, makes the major contribution to the interaction with fibulin-2.

Unlike CCP2, CCP1 is not compactly folded—CCP1 and CCP2 are both expressed as soluble proteins in good yield, have appropriately paired disulfides, and yield similar far-UV CD profiles. On the other hand, they have contrasting biophysical properties. CCP2, which has a sequence more typical of CCP modules, is compactly folded. It is stable even at relatively high temperature or denaturant concentration, and its disulfide bridges and hydrophobic side chains are largely buried and generally not accessible to solvent (only a small level of ANS binding was detected), giving rise to a very strong near-UV negative ellipticity. Its three-dimensional structure was solved, yielding the first structural information for any part of the GABAB receptor. The structure of CCP2 is similar to the structure of CCP1 and, as in the present case, had no significant effect upon three-dimensional structure. The variable levels of $N$-glycosylation at Asn$^{83}$ were anticipated from previous studies (22), but the presence or absence of glycosylation had no effect on the structure as judged by NMR or its stability according to DSC (data not shown). Asn$^{23}$ (in the seven-residue N-terminal extension; not present in the previously reported construct) was found to be $N$-glycosylated in this study. The presence or absence of this extension (with or without its glycan) had no significant effect upon structure or stability (data not shown). Nonetheless, large glycans are undesirable in NMR studies, so the carbohydrates were trimmed with endoglycosidase H$_{t}$ to leave single GlcNAc residues. The high yield of protein expression and the formation of disulfides in the expected Cys-I–Cys-III and Cys-II–Cys-IV pattern are both consistent with proper processing by the secretory pathway. In previous studies (50), other CCP modules (e.g., from complement receptor type 1) were mutated to remove $N$-glycosylation sites prior to successful expression and structure determination. The possibility that the addition to the core sugars of more complex, branched saccharides (lacking in P. pastoris) is a specific structural requirement in CCP1 seems very unlikely.

Recombinant CCP Fragments from the GABAB Receptor Are Able to Bind to Fibulin-2—A useful criterion of the authenticity of a recombinant protein is its functional activity. Based on a preliminary yeast two-hybrid study, the putative CCP modules of the GABAB R1α subunit were reported to interact with the extracellular matrix protein fibulin-2 (17–20). In this study, this interaction was shown using pull down-type assays for the first time, thus confirming an intriguing interaction between the GABAB receptor and an extracellular matrix protein. That the CCP12 fragment expressed in P. pastoris could be used in affinity purification of fibulin-2 is also highly significant because this confirms that the material expressed in yeast retains one of the functional properties of the intact receptor. The previously reported yeast two-hybrid experiments were negative for CCP2 binding to fibulin-2 (18, 19). The results presented here confirm that CCP1, in both the native and recombinant forms, makes the major contribution to the interaction with fibulin-2.

The functional relevance of CCP1 interaction with fibulin-2 is still unknown. Fibulins are present in the central nervous system and are known to have roles in development (61). The GABAB R1α (two CCP modules) and GABAB R1c (possessing only CCP1) isoforms are more highly expressed in fetal brain compared with GABAB R1b (13), and this suggests a possible role for the interaction between CCP and fibulin-2 in human brain development. An interaction of the GABAB receptor (although not involving the CCP modules) with another extracellular matrix protein, tenascin-R, has also been reported (21). Tenascin-R was shown to modify GABAB receptor activity. Efforts to detect modulation of GABAB receptor activity by fibulin-2 are under way.

**DISCUSSION**

Preparation of CCP Modules from GABAB R1α—The work represents an additional example of the successful use of P. pastoris to express CCP modules in useful yields. Daily harvesting was required because CCP1 and CCP12 are susceptible to rapid cleavage (after Lys$^{51}$) (data not shown). The presence of oxidized methionine was previously reported in recombinant CCP modules from complement receptor type 1.
FIG. 7. Solution structure of GABA<sub>B</sub> R1a CCP2. A total of 24 structures are shown as backbone traces overlaid on all C-α atoms from Cys<sub>99</sub> to Cys<sub>156</sub>. a, structures of the trans-form. b, structures of the cis-form. See Table I for r.m.s.d. c, Molscript (71) representation of the closest-to-mean structure of the trans-form. Assignment of β-strands was based on a consensus among members of the ensemble (Procheck-NMR (72)) and annotated according to Henderson et al. (58). Cysteines and tryptophan are shown in ball-and-stick representation. d, C-α trace superposition (52) of all solved CCP module structures (trans-CCP2 and cis-CCP2 in red and 25 others in cyan). e, view of trans-CCP2 (red) and cis-CCP2 (green) superimposed (all equivalent C-α atoms (52)) on the structure they resemble most closely, that of the third module of DAF (DAF~3; blue) using Multiprot. Images in d and e were produced using RasMol (73).
Disorder Is Important for Function?—Numerous examples of functionally competent proteins with a high content of native disorder exist intracellularly, and several extracellular examples are now recognized (65, 66). The common occurrence of native disorder has been recognized in the availability of the software tool PONDR (Predictor of Natural Disordered Regions) (67–69). The CCP1 and CCP2 sequences were submitted to PONDR. The region from Arg67 to Val77 of CCP1 was predicted as disordered. This corresponds (according to a multiple sequence alignment (22)) to strands E and F in CCP modules of known structure. PONDR did not predict disorder in CCP2, and no or very little disorder was predicted in the CCP modules of Vaccinia virus complement control protein and membrane cofactor protein. Disorder might also be expected in the region of CCP1 corresponding to the hypervariable loop in other CCP modules, which is uncommonly long. In this respect, it is interesting to note that Lys51, where CCP1 proteolytic cleavage occurs, is predicted to be part of the hypervariable loop. However, in the absence of a backbone assignment, it is difficult to infer from the experimental data the precise extent of disorder in CCP1.

Despite the relative lack of precedent for a disulfide-stabilized extracellular protein domain with a high degree of native disorder, we do know from studies of dynamics in other examples that CCP modules are relatively flexible on a range of time scales. For example, in no CCP module studied so far are there any amide protons that persist for more than a few hours after dissolving a protonated CCP module in D2O. Furthermore, some CCP modules display a wide range of 1H-15N NOEs and backbone 13N order parameters throughout their sequence, indicative of motion on a fast time scale. They also contain a high proportion of residues undergoing slow (millisecond-to-microsecond) conformational rearrangements. Another N-terminal CCP module (the first CCP module of membrane cofactor protein) with several binding partners was found to have more mobility than an internal module (the 16th CCP module of complement receptor type 1) (70). Therefore, it is conceivable that a spectrum of mobilities exists among CCP modules and that CCP1 of the GABAB receptor lies at one extreme. Proteins with high levels of native disorder are thought of in terms of the advantage such conformational plasticity affords in terms of...
displaying a range of affinities for a variety of ligands. In this respect, it is interesting to note that our yeast two-hybrid screens identified the C-terminal domain of all five members of the fibulin family. The exact purpose of the N-terminal CCP modules that differentiate GABA\(_B\) R1a from GABA\(_B\) R1b is unknown, but the results presented here suggest that they
could participate in protein-protein interactions with several extracellular matrix proteins.

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REFERENCES
2. Bowery, N. G., Bettler, B., Froestl, W., Gallagher, J. P., Marshall, F., Raiteri,