

Monomeric state and ligand binding of recombinant GABA transporter from *Escherichia coli*

Xiao-Dan Li^{a,1}, Anthony Villa^{a,2}, Colleen Gownley^{a,3}, Myong Jin Kim^{a,4}, Jinmei Song^a, Manfred Auer^a, Da-Neng Wang^{b,*}

^aSkirball Institute of Biomolecular Medicine, New York University Medical Center, 540 First Avenue, New York, NY 10016, USA

^bDepartment of Cell Biology, New York University Medical Center, 540 First Avenue, New York, NY 10016, USA

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Abstract The γ -aminobutyric acid (GABA) transporter from *Escherichia coli* was homologously overexpressed and purified to homogeneity with a yield of 1.0 mg per liter culture. The purification procedure consists of a cobalt affinity column, proteolytic cleavage of His- and myc-tags, and size-exclusion chromatography. The purified transporter exists as a monomer in FOS-Choline 12 detergent, with a Stokes radius of 45 Å for the protein–detergent complex. In detergent solution the protein binds substrates, as indicated by tryptophan fluorescence quenching. Its dissociation constants (K_d) for GABA, muscimol and nipecotic acid are 13.8, 13.3 and 27.9 μ M, respectively. This protein preparation provides ideal starting materials for future biochemical, biophysical and structural studies of the GABA transporter. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: γ -Aminobutyric acid transporter; γ -Aminobutyric acid; Neurotransmitter; Transporter; Membrane protein; *Escherichia coli*

1. Introduction

γ -Aminobutyric acid (GABA) is a major neurotransmitter in the vertebrate central nervous system [1]. In bacteria, it serves as a source for carbon and nitrogen under nitrogen-limiting conditions [2,3]. GABA uptake in *Escherichia coli* is catalyzed by the membrane transport protein, GabP. The

translocation of GABA and its analogues by GabP is driven by the membrane potential [4]. Inhibitors of GabP fall into three categories, open chain analogues, planar analogues, and planar heterocyclic compounds without a carboxyl group [5]. Some of these inhibitors are also substrates for they can be translocated across the membrane by GabP under appropriate conditions [6].

E. coli GABA transporter is a member of the amino acid/polyamine/organocation (APC) transporter superfamily [7–9]. Proteins in this family are responsible for the uptake of various amino acids and related compounds. They are widely distributed in prokaryotic and eukaryotic organisms. Many new APC proteins have recently been identified in newly sequenced genomes, making the amino acid/polyamine/organocation (APC) transporter superfamily one of the two largest families for secondary active transporters [9–11].

As a prototype of the APC transporters, the GabP protein's transport characteristics and structure–function relationships have been extensively studied by King and co-workers [5,6,12–16]. The GabP gene has been cloned [3], sequenced [4] and expressed in *E. coli* under *lac* control [5]. The protein consists of 466 amino acids and is believed to have 12 transmembrane α -helices, with both N- and C-termini facing the cytosol. Such a topology model is supported by *LacZ* and *PhoA* fusion experiments [14]. A conserved consensus amphipathic region (CAR) in the sequence has been identified for members of the APC family, ranging from bacteria to mammals [16]. The CAR region in GabP extends from the middle of transmembrane helix 8, through its connecting loop, into helix 9. Together with helix 4, the CAR sequence is thought to form the substrate translocation pathway. Mutations of polar amino acids in this region have resulted in a deleterious effect on GABA transport activity [16]. Moreover, Cys-300 at the beginning of the CAR sequence is particularly important, since its binding to certain thiol reagents abolishes the GABA transport completely [15].

Direct structural information from two- or three-dimensional crystals of the protein is needed to understand the substrate specificity and to discover the translocation pathway of GabP. Likewise, proteoliposomes reconstituted from the GABA transporter are necessary for studying its transport kinetics in detail. Both goals will require purified and stable GabP protein. In this paper, we report homogeneous overexpression and purification of the *E. coli* GABA transporter. The oligomeric state of the protein in detergent solution and its binding to substrates/analogues have been studied.

*Corresponding author. Fax: (1)-212-263 8951.

E-mail: wang@saturn.med.nyu.edu

¹ Present address: Department of Microbiology, HHSC 1216, College of Physicians and Surgeons, Columbia University, 701 W 168th Street, New York, NY 10032, USA.

² Present address: Progenics Pharmaceuticals Inc., 777 Old Saw Mill River Road, Tarrytown, NY 10591, USA.

³ Present address: R.W. Johnson Pharmaceutical Research Institute, Route 202, P.O. Box 300, Raritan, NJ 08869, USA.

⁴ Present address: ImClone Systems, Inc., 180 Varick Street, New York, NY 10014, USA.

Abbreviations: BCA, bicinechonic acid; DDM, dodecylmaltoside; FC-12, FOS-Choline 12; FPLC, fast-performance liquid chromatography; GABA, γ -aminobutyric acid; GabP, γ -aminobutyric acid transporter; HPLC, high-performance liquid chromatography; muscimol, 3-hydroxy-5-aminomethylisoxazole; nipecotic acid, 3-piperidine carboxylic acid; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate

2. Materials and methods

2.1. Materials

Plasmid pBAD was purchased from Invitrogen (San Diego, CA, USA), restriction enzymes from New England Biolabs (Beverly, MA, USA), cloning kits from QIAGEN (Chatsworth, CA, USA), and detergents from Anatrace (Maumee, OH, USA). All other chemicals were from Sigma (St. Louis, MO, USA) and were of analytical grade or better.

2.2. PCR cloning

The GabP gene was amplified by PCR, using chromosome DNA purified from *E. coli* DH5 α cell lysate as the template. Forward and reverse primers, designed according to the DNA sequence of the protein [4], were: CATGCCATGGGGCAATCATCGCAAC (25-mer), and CCAAGCTTTTATCAGCGCGTATTATGAACG (31-mer), respectively. The underlined regions represent the homologous sequences. The PCR product was cloned into the pBAD vector (Invitrogen, Carlsbad, CA, USA) [17], using *Nco*I and *Hind*III restriction enzymes. The vector contained a C-terminal *myc*-epitope and His₆-tag. The GABA transporter was expressed in the *E. coli* LMG194 strain following arabinose induction.

2.3. Optimization of DNA construct

Expressed GabP protein was purified by cobalt affinity chromatography. Various proteases (trypsin, chymotrypsin, subtilisin, elastase and thermolysin) were mixed individually with GabP at ratios between 1:100 and 1:1000 (w/w) and incubated at 4°C. Samples were withdrawn at various time intervals and subsequently analyzed by SDS-PAGE. When a candidate for the rigid protein core of GabP was found, the protein sample was subjected to N-terminal peptide sequencing. Molecular mass of the digestion product was measured by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry [18,19], in Dr. T. Neubert's laboratory. The information of N-terminal sequence and molecular mass was subsequently used to design a new DNA construct containing a thrombin site to facilitate the production of the protein core. Based on the plasmid from the first round, a second round of cloning was done using a new reverse primer: CCAAGCTTGCTGCCTCTGGG-CACCAAATTATGAACGGGTGTTTTTGCCA (50-mer).

2.4. Cell culture and overexpression

A 4 l flask containing 1.5 l LB medium with ampicillin was inoculated with 15 ml overnight culture, and grown at 37°C for 2 h to an OD₆₀₀ of 0.5–0.6. Following induction with 0.2% arabinose, the cells were grown for 2 additional h at 25°C, and harvested at an OD₆₀₀ of 1.0–1.1 by centrifugation at 7000 \times g for 12 min.

2.5. Purification and thrombin digestion

Fresh cell pellet was resuspended at 5 ml/g in TBS buffer (50 mM Tris, pH 8.0, 400 mM NaCl) containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail (Sigma). This was followed by three cycles of French Press at 18 000 psi. The suspension was centrifuged at 15 000 \times g for 20 min to remove remaining unbroken cells. The membrane was harvested by ultracentrifugation at 100 000 \times g for 2.5 h and stored at –20°C.

All purification steps were carried out at 4°C. Membrane pellet was homogenized and solubilized for 30 min in buffer containing 50 mM Tris/HCl, pH 8.0, 300 mM KCl, 1 mM fresh PMSF, 20% glycerol, 1.0% dodecylmaltoside (DDM) and protease inhibitor cocktail, at a ratio of 10 ml of buffer per gram of membrane. The insoluble portion was removed by ultracentrifugation at 100 000 \times g for 30 min. DDM solubilized membrane was incubated with Co²⁺-Talon resin (Clontech, Palo Alto, CA, USA) at 0.5 ml per gram of membrane for 2.5 h. The resin was washed twice with 10 column volumes of buffer with 50 mM potassium phosphate, pH 7.0, 300 mM KCl, 20% glycerol, and 0.1% FOS-Choline 12 (FC-12), containing 0 and 25 mM imidazole, respectively. GabP was eluted in three steps, each with two column volumes of the same buffer, containing 250, 500 and 750 mM imidazole, respectively. EDTA at 0.5 mM was added to the protein samples immediately after the elution to minimize proteolysis. The His-tagged protein was detected by Coomassie stained SDS-PAGE and, when necessary, by Western blot analysis using INDIA HisProbe-HRP (Pierce, Rockford, IL, USA). Following overnight thrombin digestion with 8 NIH U/mg (ICN, Costa Mesa, CA,

USA), the first two elutes were combined and loaded onto a Superdex 200 size-exclusion column on FPLC (Amersham-Pharmacia, Piscataway, NJ, USA). The column was pre-equilibrated with 10 mM potassium phosphate, pH 7.0, 50 mM KCl, 0.5 mM EDTA, 3 mM NaN₃, 20% glycerol and 0.1% FC-12. It was run in the same buffer at 0.1 ml/min, and fractions were collected at 0.3 ml/fraction. The homogeneity of purified GabP was checked by MALDI-TOF mass spectrometry, and protein concentration was determined by the Micro-BCA assay (Pierce).

2.6. Determination of Stokes radius of GabP by size-exclusion chromatography

The oligomeric state of GabP in FC-12 detergent was determined by analytical size-exclusion chromatography [20]. Purified GabP was loaded onto a Shodex SW804 size-exclusion column on HPLC (Waters, Milford, MA, USA), in buffer containing 50 mM Tris pH 8.0, 200 mM Na₂SO₄, 3 mM NaN₃ and 0.05% FC-12 of DDM. The Stokes radius of the GabP-detergent complex was determined using soluble proteins as references [21,22]. The association state of GabP in other detergents was analyzed at a concentration 0.2% above their corresponding critical micellar concentrations, using protein sample purified in 0.1% FC-12.

2.7. Substrate binding measured by tryptophan fluorescence quenching

The binding of GabP to substrates/analogues in FC-12 solution was measured by tryptophan fluorescence quenching [23,24]. Excitation wavelength of 283 nm and emission range 295–425 nm were set on a FluoroMax-2 fluorometer (Jobin-Yvon-Horiba, Edison, NJ, USA). The assay was performed using a protein concentration of 1 μ M in a 3 ml cuvette. Following establishment of the buffer baseline at 20°C, fluorescence data were collected while the protein sample was titrated with ligand. The dilution effects of the ligand addition (less than 2% of the total volume) were corrected for during the data processing stage. Fractional changes in fluorescence quenching at 335 nm versus ligand concentration were plotted. Curve-fitting was done using a logarithm function. The binding of the following substrates/analogues was measured: GABA, 3-hydroxy-5-aminomethylisoxazole (muscimol) and 3-piperidine carboxylic acid (nipecotic acid) (Sigma).

3. Results

3.1. Optimization of the protein construct

Following transformation with the pBAD vector containing the GabP gene and a *myc*- and a His-tag, single colonies of *E. coli* LMG194 strain were screened for GabP expression after arabinose induction. Colonies with the right plasmid were selected for cell culture, followed by protein purification by Co²⁺-Talon affinity chromatography. To identify a rigid protein core for future crystallization experiments [25], purified GabP protein was subjected to proteolytic treatment at 4°C. Such a protein core was found following 30 min trypsin digestion at an enzyme-to-protein ratio of 1:500, as shown in Fig. 1A. N-terminal peptide sequencing of the proteolytic fragment produced a sequence of GQSSQPHELG, showing that the methionine residue was cleaved off posttranslationally, as often occurs with proteins overexpressed in *E. coli* [19]. A molecular mass of 50 947.7 Da was obtained for the fragment by MALDI-TOF mass spectrometry (Fig. 1B), which was in agreement with the predicted mass for sequence G2-R466, 50 948.9 Da. A thrombin cleavage site was thus cloned in between residue T465 and the *myc*-tag, using the second reverse primer (50-mer). Following thrombin digestion, the new construct was resistant to further limited proteolysis by proteases, and was therefore concentrated on in later experiments.

3.2. Overexpression and purification of GabP

E. coli GabP protein with a thrombin cleavage site, C-ter-

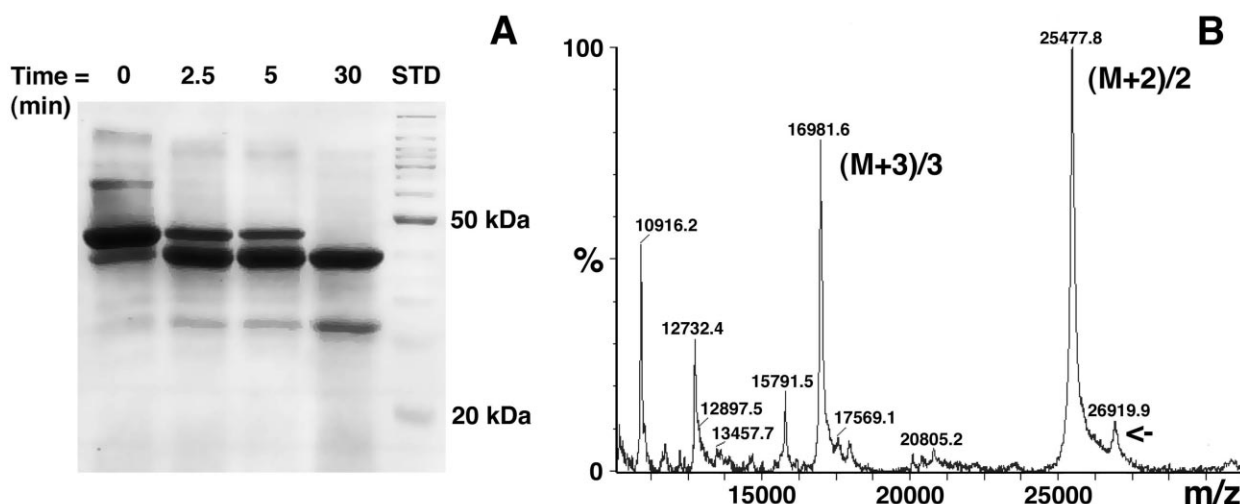


Fig. 1. DNA construct design for GabP overexpression. A: The GabP protein (without a thrombin site) purified by Co^{2+} -Talon affinity chromatography, was incubated at 4°C with trypsin at a ratio of 1:500 (w/w). Samples were withdrawn at various time points and analyzed by 12% SDS-PAGE. B: The sample of 30 min digestion was analyzed by MALDI-TOF mass spectrometry. The major double- and triple-charged peaks yielded a molecular mass of 50 947.7 Da, corresponding to amino acids 2–466 ($M_w = 50\,948.9$ Da). The small peak indicated by arrow was of the doubly-charged, undigested protein, with a measured mass of 53 837.8 Da, whereas the molecular weight predicted from sequence was 53 839.0 Da. The accuracy for the mass measurements was 0.002%. STD: molecular weight standards.

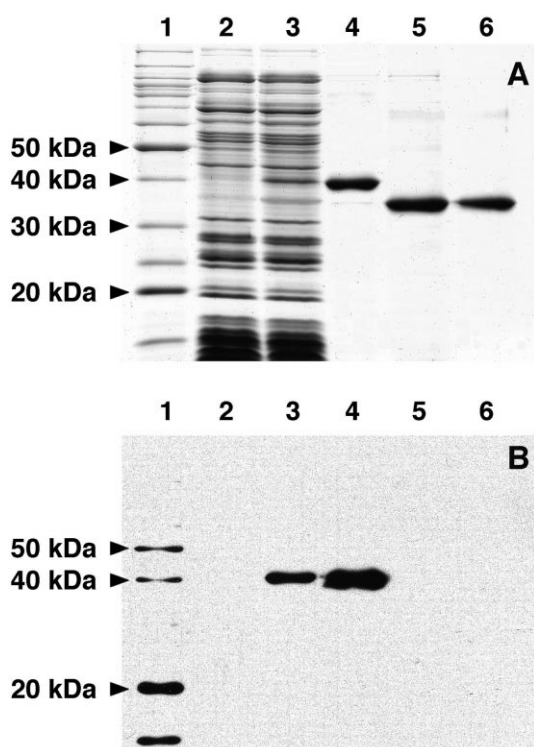


Fig. 2. Homologous overexpression and purification of *E. coli* GABA transporter. A: 10% SDS-PAGE. B: Western blot. Molecular weight standards (lane 1). DDM-solubilized membranes from cells before (lane 2) and after arabinose induction (lane 3) show overexpression of GabP. Expressed GabP protein (with a thrombin site) was purified by Co^{2+} -Talon affinity chromatography in 0.1% FC-12 (lane 4). Following the removal of His- and myc-tags by thrombin treatment (lane 5), the sample was purified further by size-exclusion chromatography (lane 6). The Western blot was performed using HisProbe.

minimal *myc*- and a His-tag, was expressed in *E. coli* LMG194 strain at a level of higher than 1 mg per liter of cell culture. Reducing the cell culture temperature from 37°C to 25°C immediately after arabinose induction was effective in minimizing degradation of the expressed protein. Approximately 90% of the cells were broken with three cycles of French Press. Expressed GabP protein was localized in the cell membrane (Fig. 2), as opposed to forming inclusion bodies, since the GabP protein was only detected in the membrane fraction after centrifugation. Though several detergents were tested, including octylglucoside and decylmaltoside, dodecylmaltoside proved to be most efficient at solubilizing the membrane and extracting GabP. Following a wash with 25 mM imidazole to remove contaminant proteins that bound non-specifically to the Co^{2+} -Talon resin, 90% of the GabP protein was eluted at 250 and 500 mM imidazole, in the presence of 0.1% FC-12 detergent. Overnight thrombin treatment at 4°C removed the His- and *myc*-tags completely, as shown by SDS-PAGE and

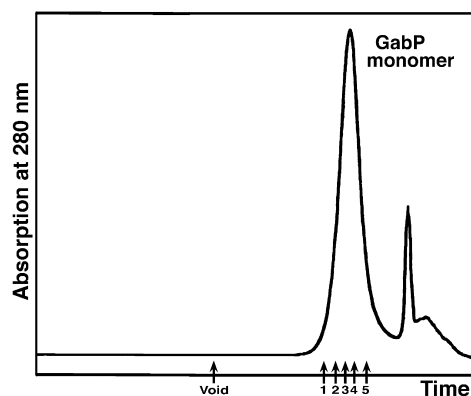


Fig. 3. Determination of oligomeric state of GABA transporter. The oligomeric state of the GabP protein in FC-12 detergent was analyzed using size-exclusion HPLC. The Stokes radius of the GabP-detergent complex was determined by comparison with soluble proteins with known Stokes radii: 1, ferritin (63 Å); 2, amylase (51 Å); 3, aldolase (46 Å); 4, albumin (35 Å); 5, ovalbumin (28 Å).

Table 1
Association state of *E. coli* GabP protein in detergent

Detergent	Oligomeric state	Detergent	Oligomeric state
Decylmaltoside	M, D, A	octylglycoside	D, A
Undecylmaltoside	M, D	nonylglycoside	D, A
Dodecylmaltoside	M	FC-10	M, D
Cymal-5	M, D, A	FC-12	M
Cymal-6	M, D	C ₈ E ₅	A
LDAO	A	C ₁₂ E ₈	A

M, monomer; D, dimer; A, aggregation.

Western blot analysis, where the GabP protein band shifts downwards from 40 to 37 kDa (Fig. 2). The digested protein was resistant to further limited proteolysis when subjected to treatment with trypsin, chymotrypsin, elastase and thermolysin. The cleaved tags and remaining contaminants were separated from GabP using a Superdex 200 column on a FPLC system, in the presence of 0.1% FC-12. MALDI-TOF mass spectrometry showed that the GabP protein after thrombin digestion was highly homogeneous. Final protein purity was estimated to be 95–98% from SDS-PAGE. Typically, 9 l of bacterial cell culture produced 18 g of cells, yielding 6 g membrane. Purification by cobalt affinity chromatography resulted in 9.3 mg of protein. Subsequent purification with size-exclusion chromatography gave a final yield of 3–4 mg of the GABA transporter.

3.3. Oligomeric state of purified GabP

The oligomeric state of FC-12 solubilized GABA transporter was analyzed by size-exclusion HPLC. The protein eluted as a single, symmetrical peak on a size-exclusion Shodex SW804 column both pre- and post-thrombin digestion. Using soluble proteins as references, the Stokes radius of GabP-(FC-12) complex was determined to be 45 ± 6 Å (Fig. 3), indicating a monomeric GabP in the detergent solution (see Section 4). The protein remained monodisperse in FC-12 for over 3 weeks at 4°C. In contrast, dodecylmaltoside was able

to preserve the monomeric state of GabP for 1 week before aggregation and precipitation occurred. Subsequent analysis of GabP in other detergents revealed aggregation of the protein or a mixture of different oligomeric states (Table 1).

We also investigated the effect of pH on the stability and association state of the protein (Table 1). GabP was monomeric over a pH range 5.0–7.5; at pH 3.5 and pH 9.5 dimers and higher oligomers were detected. In addition, glycerol was effective in stabilizing GabP, as with other membrane proteins (Boulter and Wang, submitted). Finally, GABA or DTT did not affect the oligomeric state of this transporter.

3.4. GabP binding to substrates/analogues

The binding of the GabP protein to GABA, muscimol and nipecotic acid in FC-12 solution was analyzed using tryptophan fluorescence quenching. The excitation wavelength was set at 283 nm to maximize tryptophan fluorescence. Changes up to 12% in tryptophan fluorescence intensities at an emission wavelength of 335 nm were observed. The dissociation constants (K_d) of GabP for GABA, muscimol and nipecotic acid, taken at 50% of the saturation of fluorescence quenching, were 13.8, 13.3 and 27.9 μ M, respectively (Fig. 4).

4. Discussion

We have successfully overexpressed, purified and characterized the γ -aminobutyrate transporter from *E. coli*. Since the LB medium used for *E. coli* cell culture is not nitrogen-limited, we assume that the endogenous GabP gene is expressed at negligible levels. A single protein species was detected by mass spectrometry following thrombin digestion and purification. Its molecular mass is consistent with that of our designed GabP construct.

Once extracted from the membrane, GabP is found to be sensitive to the detergent used for purification. Though dodecylmaltoside can extract GabP efficiently from the membrane, FC-12 is preferred for its ability to stabilize the protein and is therefore used in both chromatography purification steps. The FC-12 detergent molecule is chemically similar to phosphatidylcholine lipid. Its micelles, therefore, probably closely resemble the biological membrane environment.

The oligomeric state of GabP protein in FC-12 detergent can be determined by comparing its Stokes radius with those of other membrane proteins. Both the Band 3 transmembrane domain and the erythrocyte glucose transporter have a similar molecular weight as well as number of transmembrane helices as GabP. The Stokes radius of the dimeric Band 3 transmembrane domain-DDM complex is 75 Å [20]. The monomeric erythrocyte glucose transporter in decylmaltoside micelle has a Stokes radius of 50 Å (Boulter and Wang, submitted). We

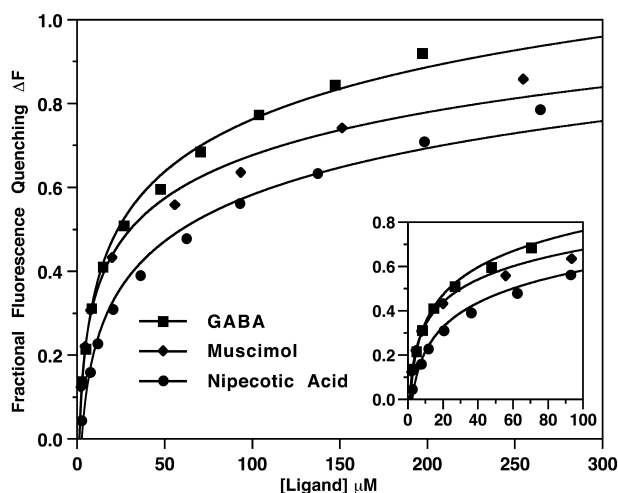


Fig. 4. GabP binding to substrates/analogues. Tryptophan fluorescence quenching of GabP in detergent solution was measured upon the addition of GABA, muscimol and nipecotic acid. Each substrate was titrated until the maximum of fluorescence quenching was reached. Fractional fluorescence changes at 335 nm were fitted with a logarithm function and plotted here. The correlation coefficient for the fitting, R-square, was 0.9–0.94 ($n=3$). A close-up of the data at low substrate concentrations is shown in the inset.

therefore conclude that GabP is a monomer in FC-12 solution.

The purified GabP monomer most likely retains its native state, as judged from its tryptophan fluorescence quenching upon the binding of three substrates: GABA, muscimol and nipecotic acid. The K_d values for these ligands measured in FC-12 solution are 13.8, 13.3 and 27.9 μ M, respectively. These agree with those determined by King and co-workers using the whole cell measurements, 12, 10 and 50 μ M [5].

It is unknown which of the 16 tryptophans in GabP contributed to the quenching of fluorescence signals upon substrate which we observed. Clearly, the full activity of the purified GABA transporter can only be assessed by transport assays following reconstitution of the protein into proteoliposomes. Nonetheless, tryptophan fluorescence provides a simple assay to analyze the integrity of transporter proteins in detergent solution [26], which is directly relevant for crystallization. The information on substrate and inhibitor binding will also be useful for designing co-crystallization experiments.

To date, few membrane transporters of amino acids or their derivatives have been overexpressed at a level greater than 0.5 mg per liter of culture [27]. The experimental protocol reported here may be used to overexpress and purify other bacterial amino acid transporters. We hope that the information on the oligomeric state and substrate binding obtained for *E. coli* GabP will help to understand biochemical properties of other APC transporters.

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