Bioorganic Chemistry **28,** 316–323 (2000)
doi:10.1006/bioo.2000.1186, available online at http://www.idealibrary.com on IDEAL®

The Unexpected Catalytic Properties of a Heterodimer of GAR Transformylase[1]

Canhui Liu, Jae Hoon Shim, and Stephen J. Benkovic[2]

Department of Chemistry, 414 Wartik Laboratory,
The Pennsylvania State University, University Park, Pennsylvania 16802

Received August 2, 2000

We have developed an efficient expression and purification protocol for a heterodimer of glycinamide ribonucleotide transformylase that was identified in incremental truncation libraries, a general combinatorial method for protein fragment complementation (M. Ostermeier, A. E. Nixon, J. H. Shim, and S. J. Benkovic, [1999], *Proc. Natl. Acad. Sci. USA* **96,** 3562–3567). This heterodimer (B13) containing both a bisection point and a deletion in conserved residues close to the active site was expressed and purified in high yield using Intein methodology. The N-terminus fragment (1-111) and C-terminus fragment (M114-212) were also expressed separately as stable proteins. When these two fragments were mixed together, they associate at a highly specific 1:1 ratio to give only the active heterodimer, B13. The activity of B13 is comparable to that of the wild type and the pH-dependent kinetics of B13 turned out to be nearly identical to those of the wild type, indicating that B13 operates in the same mechanism as the wild type. This result demonstrated that cutting within conserved regions is a viable domain separation and confirmed the generality of using incremental truncation for protein fragment complementation.

INTRODUCTION

Our group has developed a general combinatorial method for protein fragment complementation that combines two protein fragments to restore activity (*1*), which involves construction of a library of all possible combinations of lengths of N-terminal and C-terminal protein fragments generated by incremental truncation. This methodology was applied to *Escherichia coli* glycinamide ribonucleotide transformylase (GAR transformylase, PurN) in order to explore all possible cleavage points that upon noncovalent recombination would restore catalytic activity. A number of active heterodimers were identified, nearly one-half with cut points around residue 60, and the other half within the conserved region of the protein, residues 106–113 (NIHPSLLP). Of particular interest was a heterodimer (B13), comprising 1-111 (13N) and M114-212 (13C) that lacks L112 and P113, yet retains activity comparable to that of the wild type *(1).* This result was quite unexpected because this structural

[1] This work was supported by the National Foundation of Cancer Research and PHS Grant GM24129 from the National Institute of Health (S.J.B.).

[2] To whom correspondence should be addressed. Fax: (814) 865–2973. E-mail: sjb1@psu.edu.

0045-2068/00 $35.00

change contiguous to the active site and in conserved residues was assumed to be destructive to enzyme function. Thus, we questioned whether B13 still operates with the same active site functional groups as the wild-type enzyme or adopts other functional groups that would compensate the unfavorable structural change. Related issues were the stability of the interaction between the protein fragments and whether the fragments could be expressed and isolated separately before their association into an active heterodimer. To investigate these questions, we developed a high expression and facile purification system for the B13 protein fragments and analyzed its kinetic and structural properties in detail.

MATERIALS AND METHODS

Vector construction. The 13N gene fragment of purN was amplified by PCR using the forward primer, 5′-GCTAGG(CTGCAG)GCATTGAGGGTCGC**ATG**AATATTG-TGGTGCTTATT-3′ (the *Pst*I site is in parentheses and the factor Xa recognition site underlined, and the start codon in bold) and the reverse primer, 5′-GGGATA(GGTAC-C)**TTA**CAGAGAAGGGTGAATGTT-3′ (the *Kpn*I site is in parentheses and the stop (antisense) codon in bold). PCR products were purified, digested with the appropriate restriction enzymes, and ligated into *Pst*I/*Kpn*I-digested pARb [plasmid pARb was constructed by ligation of a synthetic oligonucleotide (sense strand, 5′-CATGGGTCT-GAACGACATCTTCGAAGCTCAGAAAATCGAATGGCACTCTGCA-3′; and anti-sense strand, 3′-CCAGACTTGCTGTAGAAGCTTCGAGTCTTTTAGCTTACCGT-GAG-5′) encoding the minimal peptide substrate for biotinylation (*2*) between *Nco*I and *Pst*I in the multiple cloning site of pAR4 (*3*)] to give plasmid pARb-13N.

The 13C gene fragment was amplified by PCR using the forward primer, 5′-CCTGAATTC(CATATG)AAATATCCCGGATTACAC-3′ (the *Nde*I site is in paren-theses) and the reverse primer, 5′-TAGGAATTC(GCTCTTCC)GCA**GCC**CTCGTC-GGCAG-CGTAGCCCTG-3′ (the *Sap*I site is in parentheses and the Gly (antisense) codon in bold). Gly residue was added in the C-terminus of the 13C to prevent *in vivo* cleavage. PCR products were treated with *Nde*I and *Sap*I and were ligated into *Nde*I/*Sap*I-treated pET-Impact (*4*) to give pET-Impact-13C.

Purification of the heterodimer B13. Plasmids pARb-13N and pET-Impact-13C were cotranformed into BL21(DE3) using $CaCl_2$ procedures (*5*). Transformants were selected on an LB plate containing 50 μg/mL kanamycin and 50 μg/mL chloramphenicol. Selected cells were grown in LB media containing the same concentration of antibiotics at 37°C to an OD_{600} of 0.5. Protein production was first induced by 0.3% arabinose at 25°C for 4 h and then by 0.5 mM IPTG at 25°C, followed by an additional overnight growth. After harvest, cells were resuspended in a column wash buffer (20 mM Tris-Cl, 500 mM NaCl, 0.1 mM EDTA, and 0.1% Triton X-100, pH 8.0) containing 1 mM PMSF, 4 μg/mL leupeptin, and 5.6 μg/mL pepstatin, and lysed by sonication. Cell debris was removed by centrifugation at 40,000*g* for 1 h and the supernatant was loaded onto a chitin column (3 $\times$ 2.5 cm). The column was washed with 10 column volumes of column wash buffer at 1 mL/min. The cleavage of the fusion protein was induced by flushing the column quickly with three column volumes of freshly prepared cleavage buffer (20 mM Tris-Cl, 50 mM NaCl, 0.1 mM EDTA, and 30 mM DTT, pH 8.0) and the column was left at 4°C overnight to complete cleavage. The heterodimer was eluted in three column volumes of cleavage buffer without DTT.

Fractions containing the heterodimer were pooled to load onto a Sephadex G-75 gel filtration column (5 × 100 cm) equilibrated in 50 mM Tris-Cl and 1 mM EDTA, pH 7.5. This column was run at 1 mL/min and fractions containing pure heterodimer were pooled and concentrated.

Purification of 13C. Plasmid pET-Impact-13C was transformed into BL21(DE3) using $CaCl_2$ procedures (*5*). Transformants were selected on an LB plate containing 50 μg/mL kanamycin and grown in LB media containing the same concentration of kanamycin up to an OD_{600} of 0.5. Protein production was induced by 0.5 mM IPTG at 25°C and cells were grown overnight. Purification was performed as described in the previous section.

Purification of 13N. Plasmid pARb-13N was transformed into TX680F', a purN, purT-auxotropic cell using $CaCl_2$ procedures (*5*). The cells were grown in LB media containing 50 μg/mL chloramphenicol at 37°C up to an OD_{600} of 0.5. Protein production was induced by adding arabinose (0.3% final concentration) and cells were grown for additional 6 h at 25°C. Cells were harvested by centrifugation, resuspended in a minimal volume of 50 mM Tris and 1 mM EDTA, pH 7.5, and lysed by sonication. Lysate was cleared by centrifugation at 40,000*g* for 1 h and supernatant was loaded onto a Sephadex G-75 column (1 mL/min). Fractions containing 13N were identified by activity assay after mixing with purified 13C.

Western blotting. Western blotting was performed using a rabbit antibody raised against *E. coli* GAR transformylase. A nitrocellulose membrane was used for blotting and BSA was used as a blocking agent. The protein bands were detected by goat anti-rabbit IgG-AP conjugate and BCIP/NBT reaction.

Mass spectrometry. MALDI mass spectrum of the heterodimer was obtained on a Voyager-DE STR mass spectrometer (PerSeptive Biosystems, Framingham, MA) at the Penn State University Facilities using a nitrogen laser (337 nm) and α-cyano-4-hydroxycinnamic acid as a matrix.

Kinetic measurements. All the kinetic mesurements were performed at 25°C in MTEN buffer [50 mM 2-(*N*-morpholino)ethanesulfonic acid, 25 mM ethanolamine, 25 mM Tris, and 100 mM sodium chloride]. The protein concentration was estimated using a BCA assay (Pierce). GAR was prepared as described previously (*6*). Activity assay and pH-dependent kinetics were carried out as described previously using 10-formyl-5,8-dideazafolate (fDDF) as a cofactor (*7*). Data were collected on an Olis Cary-14 spectrophotometer and processed as described previously (*7*).

In vitro association of 13N and 13C. Various amounts (1–80 nM) of 13N were mixed with 20 nM of 13C in assay mixtures or vice versa, and initial rates were measured under saturating condition. The actual concentration of 13N was estimated by densitometry of SDS-PAGE separation of the pooled gel-filtration fractions.

RESULTS AND DISCUSSION

Expression and purification of 13N and 13C. The 13N gene fragment was subcloned into a pARb vector that encodes a sequence for biotinylation and an Xa cleavage recognition site. This strategy is to express 13N as a fusion protein with a biotinylation sequence on its N-terminus for purification on an avidin affinity column followed by N-terminal cleavage by factor Xa after purification. Upon induction, however, the

biotinylation sequence was found to be cleaved intracellularly to release 13N. A G-75 column was then used to remove most of the impurities. The final purity of 13N was determined by gel scanning of Coomassie-stained SDS-PAGE gel to be about 30% (Fig. 1).

The 13C gene fragment was subcloned in a pET-Impact vector and expressed as a fusion protein with Intein-CBD. After purification on a chitin column, its removal by DTT cleavage, and G-75 column chromatography, the purity of 13C was over 98% and the average yield was about 1–2 mg/L culture (Fig. 1).

The most efficient way to prepare B13 turned out to be coexpression of 13N and 13C. The 13N-pARb and 13C-pET-Impact were cotransformed into BL21(DE3) cells. A high yield of heterodimer was achieved, when 13N was induced first with arabinose for 4 h and then 13C was induced with IPTG. Inducing both proteins at the same time gave only one-third the production of this sequential induction. It appears that the expressed 13N complexes with the emerging 13C fragment of the fusion protein in the cell, and the complex remains intact throughout the purification process (Fig. 2). The purity of the heterodimer after chitin column and DTT cleavage was about 75%. Further purification on a gel-filtration column yielded over 98% pure protein with an average yield of 4–6 mg per liter culture (Fig. 1). The reason for a lower yield of the heterodimer when both fragments were induced simultaneously might be the greater susceptibility of 13C to proteolysis than the fully assembled complex.

SDS-PAGE, Western blotting, and mass spectrometric (Fig. 1) analyses confirmed the production of B13 as the intact 1:1 heterodimer consisting of the expected molecular weights, 12.1 kDa for 13N and 11.2 kDa for 13C. Interestingly, the higher molecular weight 13N appears to travel faster on an SDS-PAGE gel than 13C.

In vitro association of 13N and 13C. Neither 13N nor 13C alone have enzymatic activity. When an increasing amount of 13N was mixed with 13C or vice versa, the

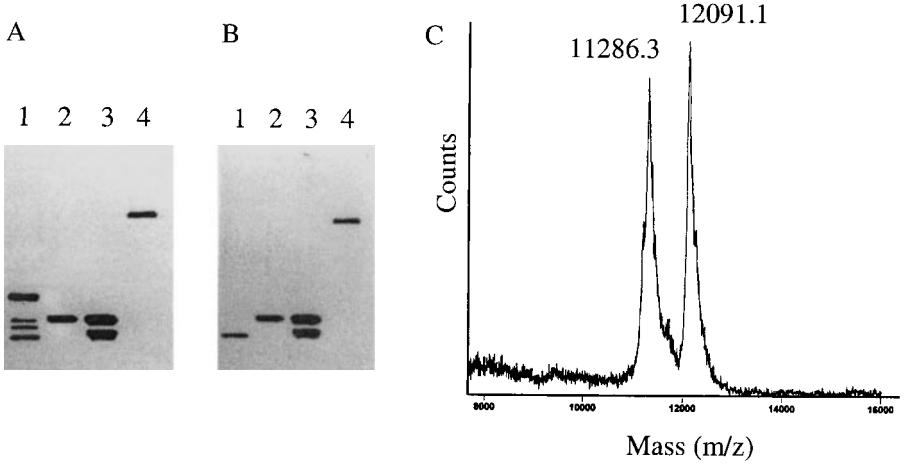

FIG. 1. (A) SDS-PAGE of protein samples after G-75 gel filtration columns. Lane 1, 13N; Lane 2, 13C; Lane 3, B13; and Lane 4, wild-type purN. (B) Western blotting of A. (C) MALDI mass spectrum of B13. Two parent molecular weights closely match the calculated MW, 11217 for 13C and 12069 for 13N.