The Optimization of Solvent Condition for NMR Studies of the Amidase Domain of Glutathionylspermidine Synthetase/Amidase from *E. coli*

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Glutathionylspermidine (GSP) synthetase, isolated from *Crithidia fasciculate* or *Escherichia coli*, has been found to be a key enzyme that participates in the first of two similar steps of trypanothione (N^1,N^8-diglutathionylspermine) biosynthesis.[1] The 70 kDa GSP synthetase has an unexpected second catalytic activity, GSP amide bond hydrolysis.[2] A fragment containing *N*-terminal 225 amino acids is ascribed to the second amidase activity.[3] Also, a major difference exists in the biochemistry of defense mechanisms of the protozoal parasites against oxidative damages from humans.[4] Unlike host cells, which use a glutathione/glutathione reductase for oxidant defense, protozoal parasites depend on trypanothione. From a pharmaceutical standpoint, interfering with the trypanothione biosynthetic pathway is attractive for antiparasitic drug design. To obtain insight into such structure-based drug design, we have attempted to determine the three dimensional structure of the amidase domain of GSP synthetase/amidase. However, during the preparation of the amidase domain of GSP synthetase/amidase, we observed that the protein suffers from aggregation below pH 7. On the other hand, the protein showed outstanding solubility above pH 7.5. But, the important resonances of the surface amide protons are likely to disappear above pH 7.5 due to the fast exchange of amide protons with solvent. To solve the protein solubility problem in conventional NMR conditions (below pH 7), we took a systematic approach to identifying solvent conditions of the amidase domain of GSP synthetase/amidase for NMR studies.

The protein was expressed from the pET-22b(+) plasmid using *E. coli* strain BL21 (DE3). ^{15}N-labeled protein was produced using M9 minimal media with 1 g/L of $^{15}NH_4Cl$ (Cambridge Isotope Laboratory) and 4 g/L of glucose. The medium also contained 0.5 g/L sodium citrate, 1 g/L $MgSO_4$, 1 mL/L trace element solution and 0.65 mL/L vitamin solution.[5] The cells were grown at 37 °C until OD_{600} reached 0.8, after which protein expression was induced for 4 hours with 0.5 mM isopropyl-d-thiogalactoside. The protein was purified to homogeneity, using Ni-NTA column (Qiagen), dialyzed against 50 mM ammonium acetate and lyophilized.

Determining the appropriate solvent conditions proceeded as follows. First, the lyophilized amidase domain of GSP synthetase/amidase was dissolved with a concentration of 24 mg/mL (1 mM) in 1 mM Tris-HCl, pH 8.0. Then 50 μL of stock protein solution, 50 μL of buffer and 50 μL of appropriate conditioning solution (Table 1) were mixed in microwell plate (Nunc) and incubated at room temperature overnight. To identify the extent of aggregation, the absorbance in each well was measured simultaneously at 620 nm, using a microplate reader (Bio-Rad, Model 550).

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Table 1. The conditions tested for solvent optimization.

Condition array	pH of buffer	Salt	Organic solvent
pH, salt	6.0, 6.5, 7.0, 7.5, 8.0	0, 150, 300 mM NaCl	–
pH, salt	6.0, 6.5, 7.0, 7.5, 8.0	0, 150 mM NaCl	5% MeOH
pH, salt	6.0, 6.5, 7.0, 7.5, 8.0	0, 150 mM NaCl	5% TFE
salt	6.0	25, 50, 75, 100, 125 mM NaPi	–
salt	6.0	100, 200, 300 mM glycine	–
salt	6.0	50, 100, 150 mM $CaCl_2$	–

Buffers were prepared from the mixed buffers of MES, MOPS and Tris. The resulting concentration of buffer in solution was 33 mM.

The protein solubility was highly dependent on pH and prominent above pH 7.5 (Figure 1). The increase in salt concentration not only reduced the pH dependency of the solubility but also improved significantly solubility below pH 7.0 (Figure 1A). The addition of organic solvents induced little effect for MeOH and decreased the solubility for TFE (Figure 1B). Divalent salts were found to be very effective solubilizers (Figure 1C). However, a zwitterionic salt (glycine) showed poor solubilizing effect (Figure 1C). These results indicate that both the ionic strength and electronic character

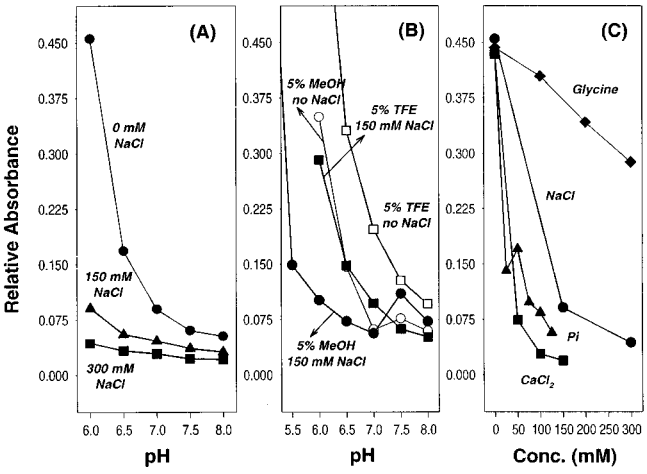

Figure 1. The optimization of solvent condition of the amidase domain of GSP synthetase/amidase. Relative absorbance indicates the extent of aggregation of the protein. (A) The concentration of NaCl was varied with 0 mM, 150 mM and 300 mM between pH of 6.0 and 8.0. Buffers were prepared from the mixed buffers of MES, MOPS and Tris. (B) 5% MeOH or TFE was added with 0 mM or 150 mM NaCl. (C) The concentration of divalent ($CaCl_2$ and NaPi), monovalent (NaCl) and zwitterionic (glycine) salts were varied at pH 6.0.

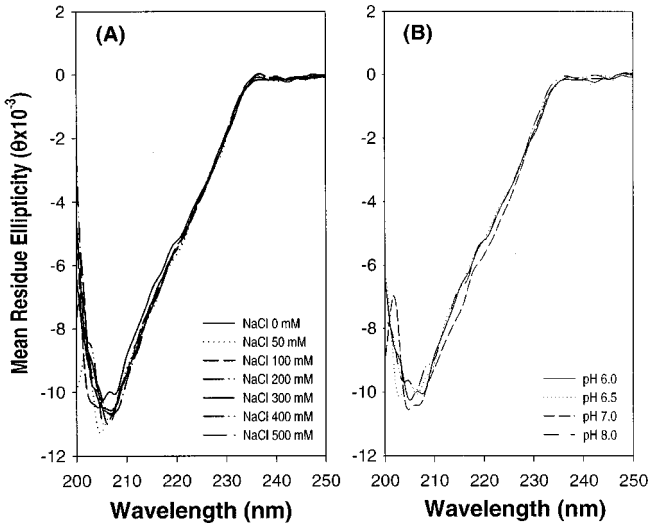

Figure 2. CD spectra of the amidase domain of GSP synthetase/amidase in varying salt concentration (A) and pH (B). Buffers were prepared from the mixed buffers of MES, MOPS and Tris. All CD spectra were measured on Jasco-720 spectropolarimeter at room temperature. The protein concentration was 100 μg/mL and cell path length was 1mm. Far-UV wavelength scans were recorded from 250 to 200 nm, with a 0.2 nm step resolution, a 50 nm/min speed, a 2 s response time, and a 1 nm bandwidth. Spectra were collected and averaged over five scans. The ellipticity results were expressed as mean residue ellipticity (MRE) in units of deg cm^2 dmol^{-1}.

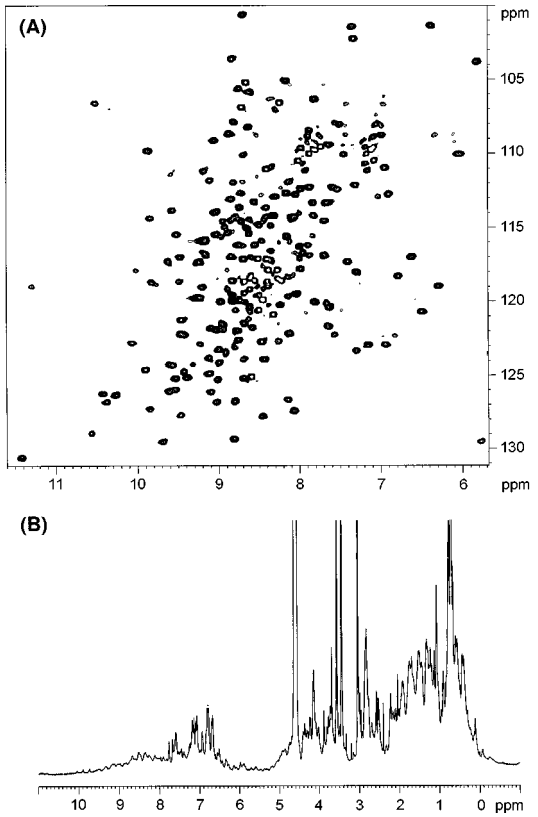

Figure 3. NMR spectra of the ^{15}N-labeled amidase domain of GSP synthetase/amidase. ^{1}H-^{15}N HSQC spectrum (A) and 1D NMR spectrum (B) were acquired in 90%H_2O/10%D_2O with 50 mM NaPi, pH 6.5, 300 mM NaCl, 3 mM DTT, 1 mM sodium azide and 0.2 mM DSS at 25°C on a Bruker 600 MHz DMX spectrometer. ^{1}H-^{15}N HSQC spectrum was acquired using ^{1}H and ^{15}N sweep widths of 12019 and 2432 Hz, respectively. For each of 256 t_1 values, 8 transients were recorded. Data were processed using the XWINNMR program.

of the salt should be considered for solubilization of the amidase domain of GSP synthetase/amidase. Circular dichroism (CD) spectra were measured to identify whether the secondary structures of protein are dependent on the pH or salt concentrations. Figure 2 shows that the secondary structure of protein is not sensitive to these conditions. Therefore, it can be concluded that the addition of salt or pH variation does not perturb the structure of the amidase domain of GSP synthetase/amidase.

Considering the optimization results, we prepared a NMR sample of the amidase domain of GSP synthetase/amidase in 50 mM NaPi, pH 6.5 and 300 mM NaCl. The protein was soluble up to 1 mM and the ^{1}H-^{15}N heteronuclear single quantum correlation (HSQC) spectrum was successfully acquired without aggregation of the protein (Figure 3A). The chemical shifts of the amide protons were well dispersed in both proton and nitrogen chemical shift axes, indicating that the amidase domain of GSP synthetase/amidase contains some β-sheet elements. The abundance of H_α proton resonances above 5 ppm in 1D spectrum also supports the presence of β-sheet elements (Figure 3B). In addition, the β-sheet content was estimated as 28% from CD results, using the CDNN program.[6]

The optimization of solvent conditions for proteins that suffer from aggregation should be carried out before extensive NMR studies. In the case of the amidase domain of GSP synthetase/amidase, the solubility of the protein improved significantly with salt addition below pH 7. The solvent condition of the amidase domain of GSP synthetase/amidase for NMR studies was optimized as a buffer of 50 mM NaPi, pH 6.5 and 300 mM NaCl. It was found that 25 distinct amide proton peaks of the amidase domain of GSP synthetase/amidase disappeared at pH 7.5, proving that the optimization of solvent condition is a pre-requisite. With the NMR sample in optimized solvent condition, several 3D heteronuclear NMR data were acquired. The resonance assignments of the amidase domain of GSP synthetase/amidase are ongoing.

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