Innovative Romanian Food Biotechnology *Vol. 12, Issue of March, 2013*

Received November 10, 2012 / Accepted February 27, 2013

RESEARCH ARTICLE

COMPARISON OF DIFFERENT MOLECULAR FORMS OF GLUTAMINE SYNTHETASE FROM *BACILLUS BREVIS* Bb G1 BY FLUORESCENCE SPECTROSCOPY

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Abstract

Glutamine Synthetase in *Bacillus brevis* Bb G1was purified from alanine grown cells (GSala) and pyruvate grown cells (GSpyr). The emission maximum, life times and quantum yield were found to be 329 nm, 5.8ns & 1.8ns and 0.097 for GSala and 320nm, 5.08ns & 1.3ns and 0.032 for GSpyr respectively. The large wavelength shift in the emission maximum, the significant differences in the shape of the spectrum, the change in fluorescence life times and the considerable differences in the quantum yields of GSala and GSpyr clearly indicated that the conformations of both these forms of the enzyme are significantly different from each other and GSpyr has a more compact structure than GSala. The shorter shifts in the spectrum of fluorescence of GSala / GSpyr compared to free tryptophan and the low quantum yield values of GSala / GSpyr indicated that the majority of the fluorescent tryptophan residues in the enzyme are buried inside the protein in a nonpolar hydrophobic microenvironment. The two life times of GSala / GSpyr indicated that the enzyme contained at least two tryptophan residues that fluoresced in two different environments.

Key Words: glutamine synthetase, fluorescence spectroscopy, conformational changes.

Introduction

Glutamine synthetase, an important enzyme of nitrogen metabolism has been suspected to play a regulatory role. The extensive regulatory property of glutamine synthetase is largely attributed because of its ability to exist in multiple forms. Bacteria under study *Bacillus brevis* Bb G1 undergoes sporulation, that is, formation of heat resistant refractile bodies called spores.

Bacteria have to take a genetic decision to express vegetative cell or spore specific genes on the basis of metabolic state of the vegetative cell. Considerable efforts have been made in the past to determine the role of glutamine synthetase in the regulation of sporulation of *Bacilli* (Reysset *et al.* 1978).

Inability to see multiple forms of glutamine synthetase in *Bacillus* species has put doubts on the role of glutamine synthetase in the regulation of sporulation (Sonenshein and Campbell, 1978). *Bacillus brevis* Bb G1, the organism used in the present study, showed a typical behavior of growth and sporulation. It fails to sporulate only if a single change of carbon source is made from an amino

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acid (e.g. alanine) to its corresponding keto acid (e.g. pyruvic acid). The earlier studies done on this organism showed that among the enzymes of nitrogen metabolism only glutamine synthetase showed differences under growth and sporulation conditions. On the basis of kinetic properties, electrophoretic behavior and radio- labeling studies, it was shown that glutamine synthetase exists in more than one form in *Bacillus brevis* Bb G1 which arise due to modification involving a phosphate group (Tiwari *et al.*, 1989). If Glutamine synthetase has to play a regulatory role, these forms should differ considerably in their conformation.

Many physicochemical techniques such as optical rotatory dispersion, circular dichroism, electron paramagnetic resonance and nuclear magnetic resonance have been applied to determine the conformational changes in biological systems (Varlan and Hillebrand, 2010). Another technique for studying changes in protein conformation is fluorescence. Fluorescence spectroscopy is among the most sensitive and one of the most versatile of the many techniques available for studying the structure and dynamics of macromolecules. This is due to the high sensitivity of various fluorescence parameters such as fluorescence spectrum, quantum yield and life time of tryptophan residues to their microenvironment and to the peculiarities of their location in protein macromolecules. Since fluorescence measurements appear to be more sensitive to molecular environment than many other physical methods, they may even indicate small structural transitions of proteins. A shift in emission maximum, life time or quantum yield gives ample evidence to indicate conformational changes in the protein molecule (Giovanni *et al*., 2008; Jennifer *et al*., 2008; Varlan *et al*., 2010; Stanciuc *et al*., 2011).

For proteins there are only three intrinsic fluors- tryptophan, tyrosine and phenylalanine. The fluorescence of each of them can be distinguished by exciting with and observing at the appropriate wavelength.

In practice, tryptophan fluorescence is most commonly studied because phenylalanine has a very low quantum yield and tyrosine fluorescence is frequently very weak due to quenching. In the present investigation glutamine synthetase was purified under sporulating (GSala) and non sporulating (GSpyr) conditions and was studied by fluorescence spectroscopy to detect possible conformational changes.

Materials and methods

Most of the chemicals used in this study were purchased from M/s Sigma Chemical Co., USA. All other chemicals used were of analytical grade. Chromatographic columns were procured from Bio Rad Laboratories, California, USA. The organism under study *Bacillus brevis* Bb G1 is a gram positive, aerobic, spore forming and rod shaped bacterium. Cells do not form chains.The organism fails to sporulate if only a single change in carbon source is made, that is, an amino acid (alanine) is replaced with corresponding keto acid (pyruvic acid). *Bacillus brevis* Bb G1 was grown in alanine / pyruvate minimal medium. Glutamine synthetase was purified by affinity chromatography using Cibacron Blue as affinity ligand attached to Sepharose 4B. Purification of glutamine synthetase was also done by DE-52 ion exchange chromatography (Tiwari *et al.*, 1989). The protein concentration for the purified enzyme, GSala was 0.089 mg / ml while the protein concentration for GSpyr was 0.050 mg /ml in 20 mM MES buffer, containing 1mM $MnCl_2$ at pH 7.0.

Fluorescence emission spectrum

The fluorescence emission spectrum of GSala and GSpyr were recorded by JY3CS spectrofluorometer at room temperature by exciting the sample with a particular excitation wavelength selected by excitation monochromator and recording the intensity distribution of emitted light with wavelengths by scanning with emission monochromator and other accessories for detection and processing of spectrum.

Quantum yield

The quantum yield of GSala and GSpyr were calculated as follows. Quinine sulphate ($5x10^{-5}$M) was used as the standard for calculating the quantum yield.

Formula used:

$$q_{sample} = \frac{F_{sample}}{F_{standard}} X \frac{OD_{standard}}{OD_{sample}} X q_{standard}$$

where:

q- quantum efficiency of sample/standard

F- area of emission spectrum of sample/standard

OD- optical density of sample/standard

Life time measurements

Decay time measurements were made with the help of an Edinburgh model 199 fluorescence time domain spectrofluorometer under single photon counting conditions and data analysis was done with a PDP 11/2 microcomputer by reconvolution method using a least-squares fitting program. The time correlated single photon counting (SPC) perhaps offers the highest sensitivity and accuracy for measuring fast fluorescence decay profiles. The reliability of the instrument was checked by using fluorescence standards, viz., anthracene in cyclohexane and rose Bengal in ethanol.

Results and discussions

Fluorescence spectrum of GSala and GSpyr

The fluorescence spectrum of GSala and GSpyr were recorded using an excitation wavelength of 284 nm. The fluorescence spectrum of GSala is shown in Figure 1. The maximum emission wavelength of the fluorescence spectrum of GSala was at 329nm and the fluorescence intensity at the maximum emission wavelength was 0.589. The fluorescence spectrum of GSpyr is shown in Figure 2. The maximum emission wavelength of the fluorescence spectrum of GSpyr was at 320 nm and the fluorescence intensity at the maximum emission wavelength was 0.486. The significant difference between the emission maximum of GSala and GSpyr indicated major conformational differences between the two forms. It is significant to note that although GSpyr molecule becomes more polar due to modification, the emission maximum shows a blue shift. This could mean the fluorophores in GSpyr have shifted to interior, that is, GSpyr is likely to be more compact molecule than GSala. A closer look at the fluorescence spectrum of GSala and GSpyr revealed that GSala have somewhat broadened peak compared to the peak of GSpyr which indicated a poorly resolved fine structure for GSala and a comparatively better resolved fine structure for GSpyr. The loss of the resolved fine structure of GSala may be due to the interaction of the fluorescent solute molecules and molecules of the solvent as GSala is at a more polar environment while the comparatively better resolved fine structure for GSpyr may be due to the less interaction of the fluorescent solute molecules and molecules of the solvent as GSpyr is at a more nonpolar environment (Rendell *et al.*, 1987).

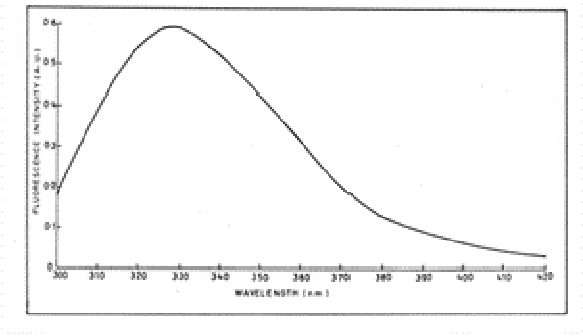

Figure 1. *Fluorescence spectrum of GS alanine at room temperature with excitation wavelength at 284nm, concentration of GSala is 0.089 mg / ml in 20mM MES buffer, containing 1mM $MnCl_2$ at pH 7.0.*

The fluorescence spectra of GSala and GSpyr were recorded using an excitation wavelength of 284nm. The fluorescence spectra showed maximum emission at 329 nm for GSala and 320 nm for GSpyr. On excitation at 280 nm, the intrinsic fluorescence of the human seminal plasma acidic protease proenzyme and *solanum tuberosum agglutimin* (STA) produced a fluorescence spectrum with a maximum emission at 340 nm and 347 nm, respectively, which is typical of proteins containing tryptophanyl residues (Surin' rut *et al.*, 1981; Doi *et al.*, 1983). The absorption maximum of phenylalanine is at 258 nm, tyrosine at 274 nm and tryptophan at 280 nm, while the emission maximum of phenylalanine is at 282 nm, tyrosine at 303 nm and tryptophan at 353 nm (Teale and Weber, 1957). The fluorescence spectra of proteins containing tryptophan had only one maximum of fluorescence, which was characteristic of tryptophan (Konev, 1967). In this case, we

observed a fluorescence maximum at 329 nm for GSala and 320 nm for GSpyr. This could be either due to averaging of fluorescence maxima of tryptophan and tyrosine or due to the presence of tryptophan in highly non polar environment. The fluorescence of tyrosine is not influenced by the environment which is in conformity by the fact that unlike tryptophan, the position of emission maximum of tyrosine at 305 nm does not vary with the environment of the fluorophore residue (Cowgill *et al.*, 1976). Therefore, the observed shifts in the fluorescence have been considered to be consequence of changes in the environment of only tryptophan residue(s) in the protein.

The shorter shifts in the emission maximum of GSala and GSpyr compared to the free tryptophan indicated that the majority of the fluorescent tryptophan residues in the enzyme GSala and GSpyr are buried inside the protein in a nonpolar hydrophobic microenvironment, supported by the studies done by several workers (Zhang *et al.*, 2007; Chilom *et al.*, 2011; Li and Wang 2011; Shinitzky *et al.*, 2011). The shorter shift in the spectrum of fluorescence of GSala and GSpyr compared to free tryptophan may be due to participation of the indole ring and hydrogen bonds in the protein molecule and / or the absence of free water inside the protein. This will result in the restrictions on vibrations of the tryptophan residues and the movement of polar molecules of the medium in the protein.

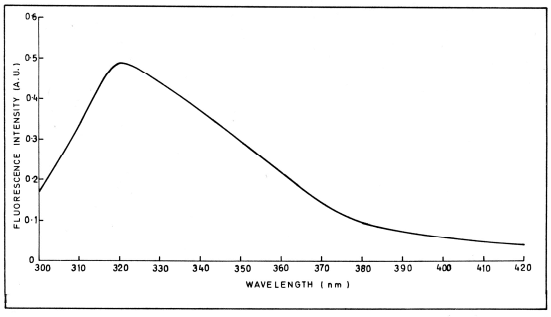

Figure 2. *Fluorescence spectrum of GS pyruvate at room temperature with excitation wavelength at 284nm, concentration of GSpyr is 0.050 mg / ml in 20mM MES buffer, containing 1mM $MnCl_2$ at pH 7.0.*

The emission maximum at 330 nm for beef liver arginase and 328 nm for bovine heart cytochrome C oxidase indicated that the majority of the fluorescent tryptophan residues are buried in a hydrophobic microenvironment (Hill *et al.*, 1986; Rossi *et al.*, 1983).

Quantum yield

Calculation of quantum yield

$$q_{GSala} = \frac{339.6}{547.7} x \frac{0.04}{0.14} x 0.546 = 0.097$$

$$q_{GSpyr} = \frac{79.6667}{547.7} x \frac{0.04}{0.10} x 0.546 = 0.032$$

The quantum yields of GSala and GSpyr were 0.097 and 0.032 respectively, significantly lower than that of free tryptophan.The low quantum yield values in GSala and GSpyr indicated that the tryptophanyl residues are deeply buried inside the enzyme in a nonpolar, hydrophobic environment (Alain *et al.*, 2003; Kelkar *et al.*, 2010; Feng *et al.*, 2011). This result is in close agreement with the studies in which it was shown that tryptophan quantum yield varied from 0.05 for γ- globulin to nearly 0.48 for bovine serum albumin (Teale, 1960).

Earlier studies showed that two discrete classes of tryptophan residues were found with different protein fluorescence maxima and quantum yields one class includes tryptophanyl residues inside the protein in a lower polar, hydrophobic microenvironment and was characterized by a short wavelength position of fluorescent maximum at 331 nm and a low quantum yield of 0.07 to 0.10.

The other class includes tryptophanyl residues on the surface of a protein in a high polar aqueous microenvironment and was characterized by a long wavelength position of fluorescent maximum at 350 nm and a quantum yield of about 0.2 to 0.25 (Konev, 1967).

Life time measurements of GSala and GSpyr

The exponential decay curve of GSala is shown in Figure 3. The life times of GSala were 5.8 ns and 1.8 ns. The exponential decay curve of GSpyr is shown in Figure 4. The life times of GSpyr were 5.08 ns and 1.3 ns. The two life times of GSala and GSpyr indicated that the enzyme contained at least two tryptophan residues that fluoresced in two different environments.

This result is consistent with the studies in which it was shown that the life times of tryptophan

fluorescence are rather short (2-5ns) and often multi exponential (Ross *et al.*, 1981; Risso *et al.*, 2010; Sarkar *et al.*, 2011).

The significant changes between the two life times of GSala and GSpyr indicated that one of the tryptophan residues in the enzyme may be relatively exposed whereas the other tryptophan residue appears to be deeply buried inside the enzyme that is in good agreement with the fluorescence studies of phosphoribulokinase which contain two tryptophans per 44KD$_a$ subunit. One of these decays with a fluorescence life time of 6.3ns and appears to be relatively exposed to solvent whereas the other tryptophan residues have a life time of 1.7 ns and appears to be buried deeper inside the enzyme (Ghiron *et al.*, 1988).

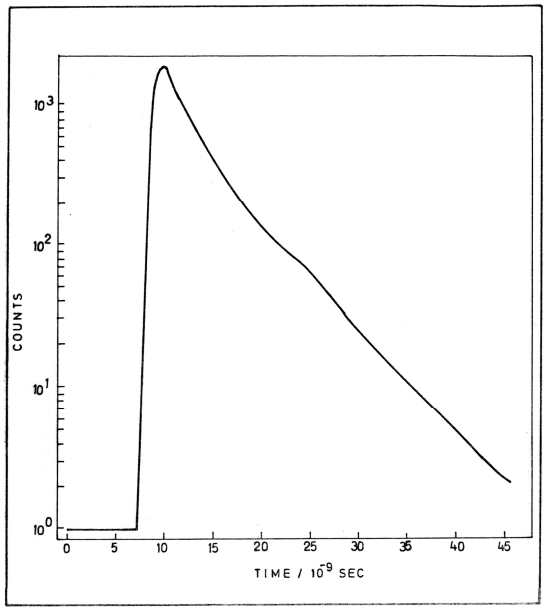

Figure 3. *The decay curve of GS alanine at room temperature (pH 7.0)*

A study on fluorescence and the location of tryptophan residues in twenty seven different kinds of proteins has revealed the existence of three discrete spectral classes. In class 1, tryptophan was buried in the nonpolar regions of the protein and had fluorescence emission maximum at 330-332nm, quantum yield 0.11 and life time 2.1ns. The class 2 had tryptophan which is completely exposed to water and showed emission maximum at 350-353nm, quantum yield 0.2 and life time 5.4ns.The class 3 showed intermediate values which is in limited contact with water and is probably immobilized by bonding at the macromolecular surface.

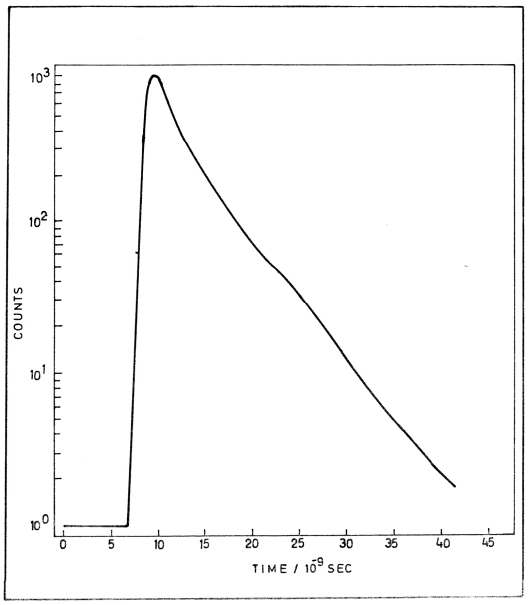

Figure 4. *The decay curve of GS pyruvate at room temperature (pH 7.0)*

This has values of emission maximum at 340-342 nm, quantum yield 0.3 and life time 4.4ns (Burstein *et al.*, 1973). Similarly, a study on the fluorescence decay kinetics of ten denatured proteins concluded that the tryptophan residues in denatured proteins can be grouped in two classes. The first is characterized by a relatively long life time of about 4ns and the second has a short life time of about 1.5ns. The emission spectrum of the group which is characterized by the longer life time is red shifted relative to the emission spectrum of the group characterized by the shorter life time (Grinwald and Steinberg, 1976).

Conclusions

Based on these results and discussions the following conclusions were made. The large wavelength shift in the emission maximum, the significant differences in the shape of the spectrum, the change in fluorescence life times and the considerable differences in the quantum yields of GSala and GSpyr clearly indicated that the conformations of both these forms of the enzyme

are significantly different from each other and GSpyr has a more compact structure than GSala. The shorter shifts in the spectrum of fluorescence of GSala / GSpyr compared to free tryptophan indicated that the majority of the fluorescent tryptophan residues in the enzyme are buried inside the protein in a nonpolar hydrophobic microenvironment.

The two life times of GSala / GSpyr indicated that the enzyme contained at least two tryptophan residues that fluoresced in two different environments. The low quantum yield values of GSala / GSpyr indicated that the majority of the fluorescent tryptophanyl residues are deeply buried inside the enzyme in a nonpolar, hydrophobic environment. This is in conformity with the earlier studies (Tiwari *et al.*, 1989) who showed that modification of glutamine synthetase in *Bacillus brevis* Bb G1 is by negatively charged group involving phosphate. That is, GSpyr is modulated with a negatively charged phosphate group. The presence of highly charged moieties at the surface is likely to make interior more compact in this enzyme form.

Hence fluorescence data, that is, emission maximum, quantum yield and shorter life times all point towards tryptophan being in a more hydrophobic region in GSpyr than the tryptophan present in GSala form of *Bacillus brevis* Bb G1.

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