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RESEARCH ARTICLE

LIGAND INDUCED CONFORMATIONAL CHANGES OF GLUTAMINE SYNTHETASE FROM BACILLUS BREVIS Bb G1 UNDER NON SPORULATING CONDITIONS - A FLUORESCENCE STUDY

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Abstract

Glutamine Synthetase from Bacillus brevis Bb G1was purified from pyruvate grown cells(GSpyr).The effect of ligands on the tryptophan fluorescence of the purified enzyme GSpyrwas investigated.With increasing concentrations of L-glutamine or glycine in GSpyr, a shift in emission maximum, change in fluorescence intensity and change in life times were observed compared to the emission maximum,fluorescence intensity and life times of GSpyr. These observations strongly support the possibility that GSpyr undergoes a conformational change on binding with ligands and each ligand produced differentconformational changes in GSpyr.Also,different concentrations of each ligand produced different protein conformations in theenzymeGSpyr.

Keywords:glutaminesynthetase, fluorescence spectroscopy, conformational changes

Introduction

Interaction of proteins with substrate or other small molecule is structurally complementary and stereo-specific. It often leads to change in the conformation of protein which has biological implication in catalysis and regulation. During the formation of an enzyme-substrate complex, the conformations of protein and substrate fit each other. The substrate causes changes in the geometry of the enzyme as it fits into the active site. A delicate orientation of catalytic groups is required for enzyme action. The substrate induces this proper orientation by the change it causes in the geometry of the enzyme. 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 (Eads and Villafranca, 1987; Varlan and Hillerbrand, 2010). A very sensitive way to study the conformational changes is monitoring the change in tryptophan fluorescence properties. It gives information on the binding of small molecules, such as substrates, coenzymes and inhibitors. In addition, it can be used to determine macromolecular conformational changes that accompany binding of these molecules or result from changes in pH or temperature. 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. The parameters of fluorescence spectroscopy such as

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fluorescence spectrum, quantum yield and life time are dependent on the molecular structure of the fluorophore. They are, however, also sensitive to the environment in or around the macromolecule carrying the fluorophore. It is this sensitivity of the emission parameters on the environment of the fluorophore which makes fluorescence spectroscopy a useful tool in the study of conformation and dynamics. When a fluorophore is attached to a macromolecule, its fluorescent properties become dependent on the local macromolecular conformation.

Thus, conformational changes which occur, for example, when a protein molecule interacts with a substrate can easily be detected by the accompanying changes in the emission parameters of the attached flurophore. 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; Abraham, 2013).A change in fluorescence intensity indicated by ligand binding to a protein can also result from local conformational changes that alter the interactions of tryptophan residues with their neighboring groups (Houston *et al.*, 2003; Varlan and Hillebrand, 2010).

Glutamine synthetase, an important enzyme of nitrogen metabolism has been suspected to play a regulatory role. The glutamine synthetase activity is modulated by a set of metabolites that include amino acid, purine, pyrimidine nucleotide, glucosamine– 6– phosphate and also by a process of modification of protein. Glutamine synthetase activity is regulated through feedback inhibition by multiple end products of glutamine metabolism. Interaction among substrates, inhibitors and manganese bound to glutamine synthetase of *E. coli* as studied by NMR relaxation rate measurements induced conformational changes in the enzyme (Eads and Villafranca, 1987). Similarly, in the present case, interaction with L-glutamine / glycine with glutamine synthetase from *Bacillus brevis* Bb G1 may undergo conformational changes to change catalytic activity or enhance interaction within the macromolecule. The enzyme GSpyr from *Bacillus brevis* Bb G1 showed negative co-operativity for L- glutamine. Also it was sensitive to inhibition by glycine (Gaur *et al.*, 1981). Therefore, different conformational changes are expected with changes in L- glutamine /glycine concentrations.

In the present investigation glutamine synthetase was purified under non sporulating conditions (GSpyr) and was studied by fluorescence spectroscopy to detect possible conformational changes that occur in the presence of L- glutamine / glycine.

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. *Bacillus brevis* Bb G1 was grown in 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, GSpyr was 0.050 mg / ml in 20mM MES buffer, containing 1mM MnCl2 at pH 7.0. The fluorescence emission spectra were recorded by JY3CS spectrofluorometer at room temperature. The 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 - anthracene in cyclohexane and rose Bengal in ethanol.

Effect of substrate / inhibitor on GSpyr

To study the effect of substrate/inhibitor on GSpyr, different concentrations of L-glutamine/glycine were prepared. In1.5ml of GSpyr, suitable amount of substrate/inhibitor was added so that the final concentrations of L-glutamine/glycine were 5, 10

and 50 mM/10, 60 and 100mM, respectively in the sample. The volume of the sample was always kept 3ml by addition of distilled water whenever required. These samples were kept for twenty minutes and then the emission spectra were taken and life times were measured.

Results and discussion

The fluorescence spectra of GSpyr and GSpyr with different concentrations of L-glutamine are shown in Figure 1. The fluorescence spectra of were recorded using an excitation wavelength of 284 nm. The fluorescence spectra showed maximum emission at 320 nm for GSpyr. On excitation at 280 nm, the intrinsic fluorescence of the human seminal plasma acidic protease proenzyme and solanumtuberosumagglutimin (STA) produced a fluorescence spectrum with a maximum emission at 340 nm and 347 nm, respectively, which is typical of proteins containing tryptophanyl residues (Surinrut *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 321 nm. 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 shift in the emission maximum of the fluorescence spectrum indicated that the majority of the fluorescent tryptophan residues in the enzyme are buried inside the protein in a non-polar 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).

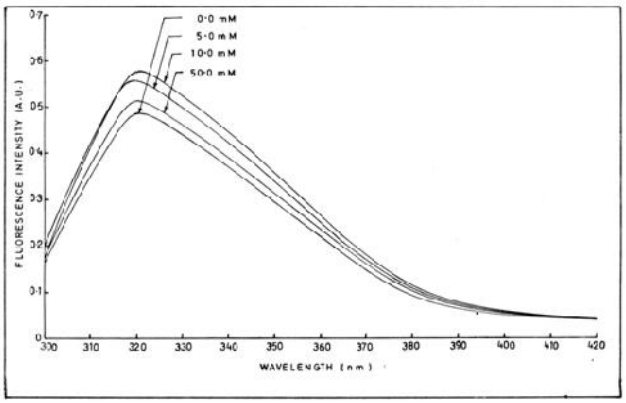

Figure 1. *Fluorescence spectra of GSpyr with various concentrations of L-glutamine at room temperature with excitation wavelength at 284nm, concentration of GSpyr is 0.050 mg / ml in 20mM MES buffer, containing 1mM MnCl2 at pH 7.0*

This shorter shift in the spectrum of fluorescence of GSpyr compared to the free tryptophan may be due to the 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. The decay curves of GSpyr and GSpyr with different concentrations of L-glutamine are shown in Figure 2.The two life times 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 (Kelkar *et al.*, 2010; Risso *et al.*, 2010; Sarkar *et al.*, 2011). The significant changes between the two life times 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 (Ghiron *et al.*, 1988).

Glutamine is the natural substrate of glutamine synthetase. In addition to acting as substrate, glutamine also acts as a modulator. The enzyme

GSpyr from *Bacillus brevis* Bb G1showedannegative co-operativity for L-glutamine (Gaur *et al.*, 1981). Therefore, different conformational changes are expected with changes in glutamine concentration. At lower concentration of L- glutamine with GSpyr, the emission maximum was blue shifted with an increase in fluorescence intensity, but at higher concentrations of L-glutamine in GSpyr the emission maximum was constant but there was an increase in fluorescence intensity compared to that of GSpyr. The fluorescence intensities increased for all the three concentrations of L-glutamine with GSpyrcompared to the fluorescence intensity of GSpyr alone. For 10mM concentration of L-glutamine with GSpyr, lifetimes T1 and T2 increased compared to that of GSpyr. For 5mM concentration of L-glutamine with GSpyr, no significant changes in lifetimes T1 and T2 were observed compared to that of GSpyr. For 50mM concentration of L-glutamine with GSpyr, life time T2 decreased with no significant change in life time T1 compared to the life times of GSpyr alone. The above changes in the emission parameters may only be due to conformational changes. Studies with fluorescence spectroscopy showed that addition of activators and substrates with proteins resulted in significant conformational changes (Hekmat *et al.*, 2008; Themiston *et al.*, 2009; Karst *et al.*, 2010; Hanske *et al.*, 2012; Orban *et al.*, 2012).

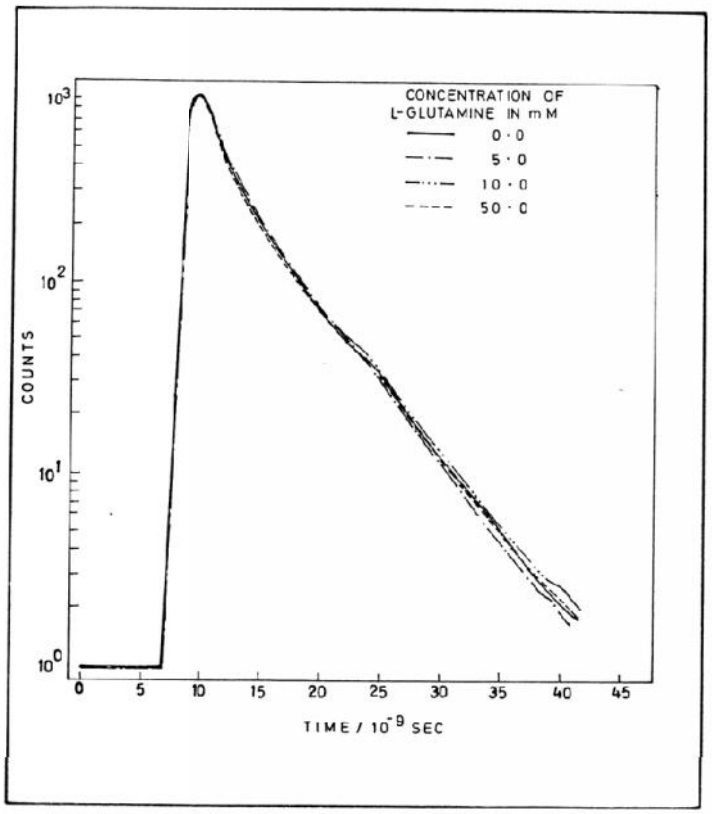

Figure 2.*The decay curves of GSpyr with different concentrations of L- glutamine at room temperature (pH 7.0)*

The addition of ATP and L-glutamate to glutamine synthetase from E.coli resulted in a large increase in fluorescence intensity with a slight blue shift of the emission maximum (Timmons *et al.*,1974).The addition of ATP and L-glutamate to glutamine synthetase from *E.coli* resulted in a large increase in fluorescence intensity with a slight blue shift of the emission maximum (Timmons *et al.*, 1974). The addition of L-glutamine to glutamine synthetaseadenyltransferasefrom *E.coli*produced increase in fluorescence intensity with a slight red shift of the emission maximum (Caban and Ginsburg, 1976). In the present study, at lower concentration of L- glutamine with GSpyr, the

emission maximum was blue shifted with an increase in fluorescence intensity, but at higher concentrations of L-glutamine in GSpyr the emission maximum was constant but there was an increase in fluorescence intensity compared to that of GSpyr. The fluorescence intensities increased for all the three concentrations of L-glutamine with GSpyr compared to the fluorescence intensity of GSpyr alone. Arsenate which activates the γ - glutamyl transferase activity by binding to an allosteric site and L-glutamate resulted in a substantial quenching of tryptophan fluorescence in bovine brain glutamine synthetase (Maurizi *et al.*, 1987). The addition of different concentrations of glutamine to QBP- Anap resulted in a large shift in emission maximum with an increase in fluorescence intensity (Lee *et al.*, 2009). The addition of glutamineto PdX1 protein resulted in a large increase in fluorescence intensity (Thomas *et al.*, 2009).

The fluorescence spectra of GSpyr, GSpyr with different concentrations of glycine, the decay curves of GSpyr and GSpyr with different concentrations of glycine are shown in Figure 3 and Figure 4 respectively.

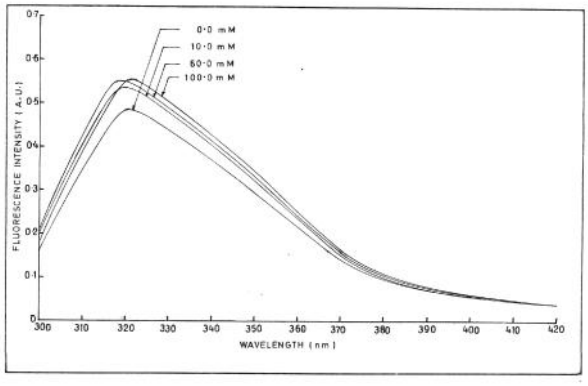

Figure 3. *Fluorescence spectra of GSpyr with various concentrations of glycine at room temperature with excitation wavelength at 284nm, concentration of GSpyr is 0.050 mg / ml in 20mM MES buffer, containing 1mM MnCl2 at pH 7.0*

Glycine is not a substrate for glutamine synthetase nor is its biosynthesis modulated through glutamine synthetase. However, studies done on fifty compounds for their ability to modulate glutamine synthetase activity, it was found that E.coli glutamine synthetase is inhibited by glycine (Woolfolk *et al.*, 1966). Earlier studies showed that the enzyme GSpyr was sensitive to inhibition by glycine (Gaur *et al.*, 1981). At 10mM and 60mM glycine, GSpyr showed a small blue shift with an increase in fluorescence intensities as compared to that of GSpyr alone. However, at 100mM glycine, GSpyr showed a small red shift with an increase in fluorescence intensity compared to the native enzyme. Also, at 100mM glycine, a 3nm red shift was observed as compared to GSpyr in 60mM glycine. The differences with respect to the effect of glutamine are obvious where no red shift is observed. The fluorescence intensities increased for all the three concentrations of glycine with GSpyr compared to the fluorescence intensity of GSpyr alone. For 10mM glycine in GSpyr no significant changes in life times were observed compared to that of GSpyr. At 60mM and 100mM glycine in GSpyr both the lifetimes T1 and T2 decreased as compared to the life times of GSpyr.

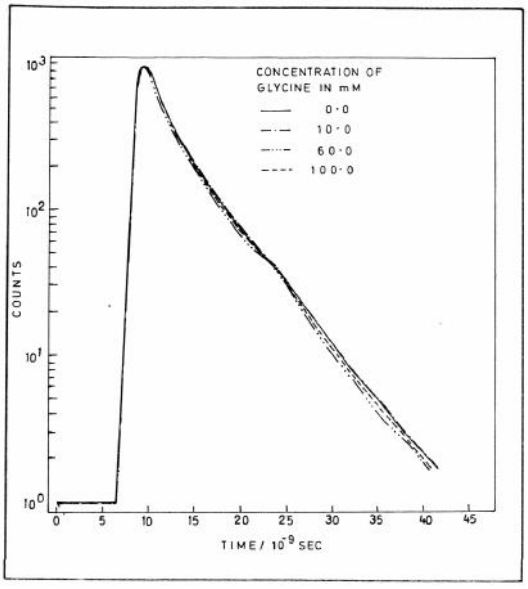

Figure 4. *The decay curves of GSpyr with different concentrations of glycine at room temperature (pH 7.0)*

The above mentioned changes in the fluorescence parameters are due to the conformational changes. Earlier studies showed that conformational changes occurred in proteins on interaction with inhibitors as studied by fluorescence spectroscopy (Haghighi *et al.*, 2005; Suresh *et al.*, 2009; Wei *et al.*, 2009; Simon *et al.*, 2012; Singh *et al.*, 2012). Conformational changes occurred in unadenylylated glutamine synthetase from *E. coli*

in the presence of various inhibitors which correlated exactly with their known inhibitory effects towards glutamine synthesis (Timmons *et al.*, 1974). Alphaketoglutarate, an inhibitor of adenylylation and an activator of deadenylylation caused a net decrease in fluorescence of glutamine synthetase adenyl transferase from *E. coli* (Caban and Ginsburg, 1976). The addition of inhibitors in monoclonal antibodies resulted in an increase in fluorescence intensity with a red shift in emission maximum (Weichel *et al.*, 2008). Changes in fluorescence intensity and shift in emission maximum were observed when α - synuclein protein interacted with Congo red and Lacmoid (Lendel *et al.*, 2009). Fluorescence intensity changes were observed with increasing concentrations of Gdn - HCl in holo and apo – aconitase (Gupta *et al.*, 2010). The fluorescence intensity decrease with red shift in emission maximum in catalase with increasing concentrations of Gdn - HCl indicated structural changes in the protein (Jiao *et al.*, 2010). Gold nano particle induced conformational changes were observed in heme protein as studied by Fourier Transform, IR and CD spectroscopy (Sahoo *et al.*, 2011). The red shift of emission maximum with decreased fluorescence intensity in fusion protein pools with LIB 38 and LIB 71 when adding Gu - HCl indicated protein unfolding and tryptophan exposure (Thomas *et al.*, 2011).The changes observed in the emission maximum, fluorescence peak intensity and life times in GSpyr in the presence of different concentrations of L-glutamine and glycine are summarized in Table1.

Table 1. *The wavelengths of emission maximum, corresponding fluorescence intensity and lifetimes of GSpyr and GSpyr with different concentrations of L-glutamine and glycine*

Sample	Emission Maximum(nm)	Fluorescence Intensity (A.U)	LifetimeT1 (ns)	LifetimeT2 (ns)
GS pyr	320	0.486	5.08	1.30
GS pyr+5mML-glutamine	319	0.555	4.96	1.30
GSpyr+10mML-glutamine	320	0.578	5.39	1.60
GSpyr+50mML-glutamine	320	0.510	5.02	0.95
GSpyr+10mMglycine	319	0.537	5.06	1.10
GSpyr+60mMglycine	318	0.548	4.73	0.93
GSpyr+100mMglycine	321	0.551	4.92	1.11

Conclusions

Based on these results and discussion, the following conclusions were made. The effects of L-glutamine and glycine on GSpyr were significantly different from each other and thus resulted in different protein conformations for each ligand. The effect of different concentrations of L-glutamine and glycine towards GSpyr were considerably different from each other. Thus, characteristically different protein conformations may be obtained at a given concentration of the ligand. The shorter shift in the emission maximum of the fluorescence spectrum compared to the free tryptophan indicated that the majority of fluorescent tryptophan residues in the enzyme are buried inside the protein in a highly nonpolar, hydrophobic microenvironment. The two life times indicated that at least two tryptophan residues in the enzyme fluoresced. One appears to be relatively exposed whereas the other deeply buried inside the enzyme. Summarizing, ligand induced conformational changes were observed in glutamine synthetase from *Bacillus brevis* Bb G1 purified under non sporulating conditions by fluorescence spectroscopic studies.

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