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Fluorescence study on ligand induced conformational changes of glutamine synthetase from *Bacillus brevis* Bb G1 under sporulating conditions

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ABSTRACT

Glutamine synthetase, an important enzyme of nitrogen metabolism, was purified under sporulating conditions (GSala). The effect of ligands on the tryptophan fluorescence of the purified enzyme GSala was investigated. With increasing concentrations of L-glutamine in GSala, a blue shift in emission maximum with an increase in fluorescence intensity and decrease in life times were observed compared to the emission maximum, fluorescence intensity and life times of GSala. With increasing concentrations of glycine in GSala, a shift in emission maximum, change in fluorescence intensity and change in lifetimes were observed compared to the emission maximum, fluorescence intensity and life times of GSala. These observations strongly support the possibility that GSala undergoes a conformational change on binding with ligands and each ligand produced different conformational changes in GSala. Also, different concentrations of each ligand produced different protein conformations in the enzyme GSala.

Key words: glutamine synthetase, fluorescence spectroscopy, conformational changes

Introduction

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 & Villafranca, 1987; Varlan & Hillerbrand, 2010a, 2010b). Another technique for studying changes in protein conformation is fluorescence. 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. The parameters of fluorescence spectroscopy such as 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; Shinitzky, 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 & Hillerbrand, 2010a, 2010b).

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The organism in this study *Bacillus brevis* Bb G1 showed a typical behavior of growth and sporulation. It fails to sporulate only if a single change of carbon source is made from an aminoacid (alanine) to its corresponding ketoacid (pyruvic acid). In the present investigation, glutamine synthetase was purified under sporulating conditions (GSala) and was studied by fluorescence spectroscopy to detect possible conformational changes that occur in the presence of various ligands.

Materials and Methods

Most of the chemicals used in this study were purchased from Sigma Chemical Co., USA. All other chemicals used were of analytical grade. Chromatographic columns were procured from BioRad Laboratories, California, USA. *Bacillus brevis* Bb G1 was grown in alanine 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 in 20 mM MES buffer, containing 1 mM $MnCl_2$ 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.

To study the effect of substrate/inhibitor on GSala, different concentrations of L-glutamine/glycine were prepared. In 1.5 ml of GSala, suitable amount of substrate/inhibitor was added so that the final concentrations of L-glutamine/glycine were 5, 10 and 50mM/10, 60 and 100 mM, respectively in the sample. The volume of the sample was always kept 3 ml 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

Glutamine is the natural substrate of glutamine synthetase. In addition to acting as substrate, glutamine also acts as a modulator. The enzyme GSala from *Bacillus brevis* Bb G1 showed negative co-operativity for L-glutamine (Gaur et al., 1981). Therefore, different conformational changes are expected with changes in glutamine concentration. The fluorescence spectra of GSala and GSala with different concentrations of L-glutamine are shown in Figure 1.

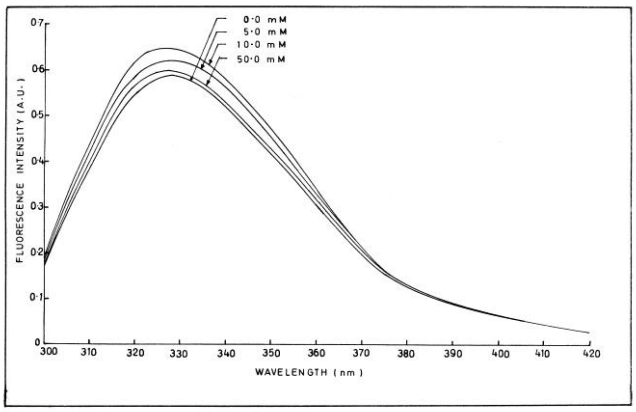

Figure 1. *The fluorescence spectra of GSala with various concentrations of L-glutamine at room temperature with excitation wavelength at 284nm, the protein concentration was 0.089 mg/ml in 20 mM MES buffer, containing 1mM MnCl2 at pH 7.0.*

For all the three concentrations of L-glutamine in GSala, the emission maximum was shifted to shorter wavelengths with an increase in fluorescence intensity compared to the emission maximum and fluorescence intensity of GSala. The emission maximum was blue shifted to 3, 5 and 2 nm for GSala with 5, 10 and 50 mM L-glutamine concentrations compared to the emission maximum of GSala and 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; Karst et al., 2010; Orban et al., 2012). 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 activators of the adenylylation reaction (ATP, L-glutamine or the *E.coli* P_{11} regulatory protein) produced an enhancement of fluorescence in the intrinsic tryptophan residue of glutamine

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synthetase adenyltransferase from *E.coli* with a slight red shift for glutamine (Caban & Ginsburg, 1976). Arsenate, which activates the γ-glutamyltransferase 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 glutamine to PdX1 protein resulted in a large increase in fluorescence intensity (Thomas et al., 2009). The fluorescence spectra of proteins containing tryptophan had only one maximum of fluorescence, which was characteristic of tryptophan. The shorter shift in the emission maximum of the fluorescence spectrum 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, supported by the studies done by several workers (Zang et al., 2007; Chilom et al., 2011). 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 (Rossi et al., 1983; Hill et al., 1986).

The decay curves of GSala and GSala with different concentrations of L-glutamine are shown in Figure 2. The lifetimes T_1 and T_2 decreased for all the three concentrations of L-glutamine with GSala compared to the lifetimes T_1 and T_2 of GSala. 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 (2-5 ns) and often multi exponential (Kelkar 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 that is in good agreement with the fluorescence studies of phosphoribulokinase, which contain two tryptophans per 44 kDa subunit. One of these decays with a fluorescence life time of 6.3 ns 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).

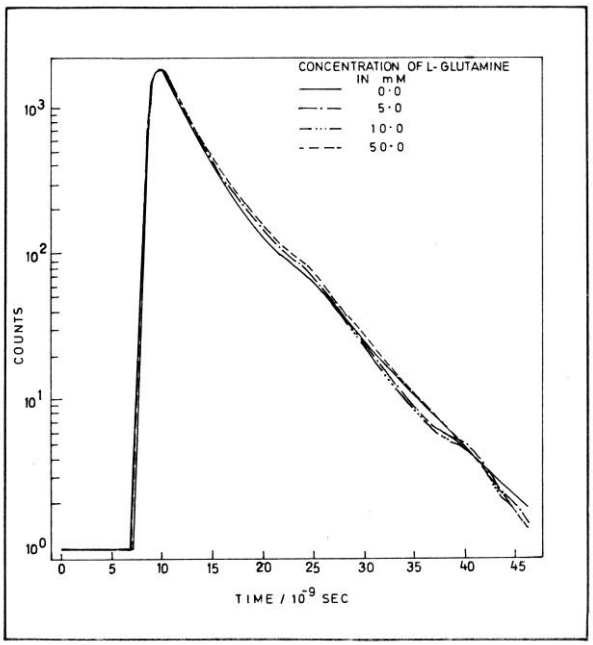

Figure 2. *The decay curves of GSala with different concentrations of L-glutamine at room temperature.*

Glycine is not a substrate for glutamine synthetase nor its biosynthesis is 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). Allosteric inhibitors, which are not substrate are also known to induce considerable changes in conformation in proteins. Earlier studies showed that the enzyme, GSala was sensitive to inhibition by glycine (Gaur et al., 1981). Therefore, different conformational changes are expected with changes in glycine concentration. The fluorescence spectra of GSala and GSala with different concentrations of glycine are shown in Figure 3.

At lower concentrations of glycine (10 and 60 mM) with GSala, the emission maximum was shifted to shorter wavelengths but at higher concentration of glycine (100 mM) with GSala, the emission maximum was shifted to longer wavelength compared to the emission maximum of GSala. The emission maximum was blue shifted 1 nm and 2 nm for GSala with 10 mM and 60 mM glycine compared to the emission maximum of GSala. However, at 100 mM glycine, a red shift of 1nm compared to the native enzyme and 3 nm as compared to GSala in 60 mM glycine was observed. The differences with respect to the effect of glutamine are obvious where no red shift is observed. For 10 mM glycine in GSala

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fluorescence intensity decreased, at 60 mM glycine in GSala fluorescence intensity increased whereas at 100 mM glycine in GSala no change in fluorescence intensity was observed compared to the fluorescence intensity of GSala.

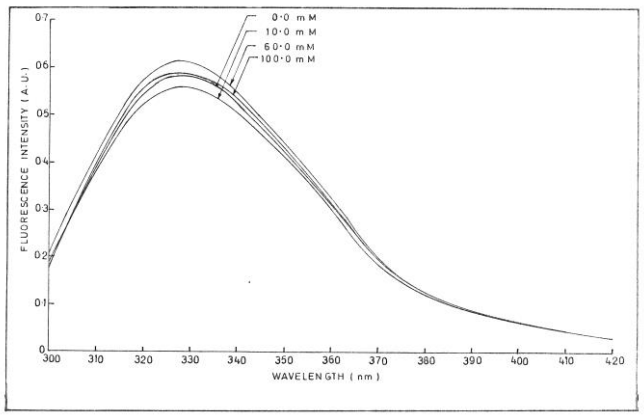

Figure 3. *The fluorescence spectra of GSala with various concentrations of glycine at room temperature with excitation wavelength at 284 nm, the protein concentration was 0.089 mg/ml in 20 mM MES buffer, containing 1 mM $MnCl_2$ at pH 7.0.*

The decay curves of GSala and GSala with different concentrations of glycine are shown in Figure 4. The lifetime T_1 increased for 10mM concentration of glycine with GSala, decreased for 100mM concentration of glycine with GSala and no significant changes were observed with 60mM concentration of glycine compared to the lifetime T_1 of GSala. The lifetime T_2 increased for all the three concentrations of glycine with GSala compared to the lifetime of GSala. The decreased life time T_1 of GSala at 100mM glycine and the increased lifetimes T_1 and T_2 of GSala in presence of 10 mM glycine may be due to differential behavior of tryptophan and tyrosine in the protein. These results, therefore, indicate that conformational changes induced by glycine are different from that of L-glutamine.

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; Wei et al., 2009; Simon 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).

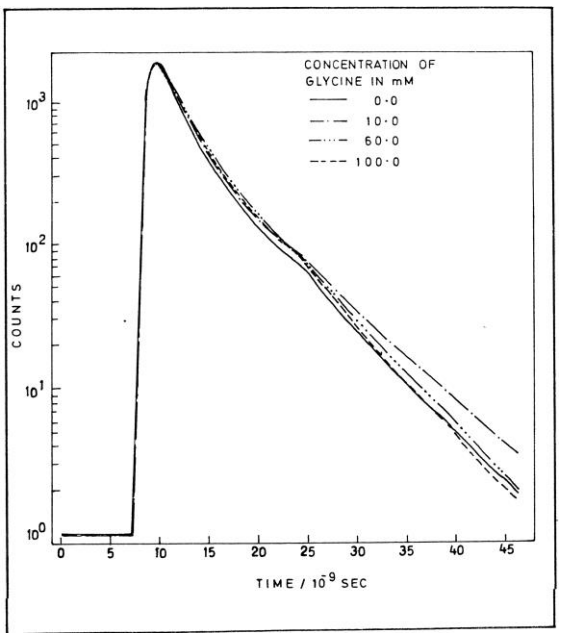

Figure 4. *The decay curves of GSala with different concentrations of glycine at room temperature.*

Alphaketoglutarate, an inhibitor of adenylylation and an activator of deadenylylation caused a net decrease in fluorescence of glutamine synthetase adenyltransferase from *E.coli* (Caban & 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 nanoparticles induced conformational changes 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 GSala in the presence of different concentrations of L-glutamine and glycine are summarized in Table1.

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

Sample	Emission maximum (nm)	Fluorescence intensity (AU)	Lifetime T1 (ns)	Lifetime T2 (ns)
GSala	329	0.589	5.80	1.80
GSala+5 mM L-glutamine	326	0.626	4.98	1.55
GSala+10 mM L-glutamine	324	0.650	4.89	1.50
GSala+50 mM L-glutamine	327	0.601	5.36	1.63
GSala+10 mM glycine	328	0.560	7.00	2.29
GSala+60 mM glycine	327	0.613	5.79	2.02
GSala+100 mM glycine	330	0.589	5.49	1.90

Conclusion

Based on these results and discussions the following conclusions were made. The shorter shift in emission maximum of the fluorescence spectrum compared to the 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 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. The effects of L-glutamine/glycine on GSala were significantly different from each other and thus resulted in different protein conformations for each ligand. The effect of different concentrations of L-glutamine/glycine towards GSala were considerably different from each other. Thus, characteristically different protein conformations may be obtained at a given concentration of the ligand. Summarizing, ligand induced conformational changes were observed in glutamine synthetase from *Bacillus brevis* Bb G1 purified under sporulating conditions by fluorescence spectroscopic studies.

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