SODIUM HYDROXIDE INDUCED CONFORMATIONALCHANGE IN 1→3-B-GLUCANS DETECTED BY FLUORESCENCE DYE ANILINE BLUE

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Abstract

Molecular conformation is considered to be an important factor in determining the biological activity of glucans, however, an easy method to detect the conformation change for glucans in solution has not been developed. We have found that the fluorescence intensity of aniline blue bound to glucan can be used to estimate the relative amount of single helix converting to triple helix during different stages of a denature-renature cycle. The native conformation for schizophyllan (SPG) (a branched β-1→3 glucan) is a rigid closed triple helix. Treatment with NaOH produces a single helix-rich preparation. We observed that aniline blue does not stain native SPG, but will stain the NaOH-treated SPG. This observation suggests that aniline blue binds only to single helix forms of SPG. The activity of renatured SPG, stabilized with aniline blue at different days, was evaluated using a glucan responsive LAL assay. The activity of LAL assay was inversely correlated with the amount of triple helix formed when SPG was renatured and stabilized over time.

Introduction

1→3-β-Glucans exhibit a variety of biological and immunopharmacological activities. The important factors for determining the biological activity are molecular weight, degree of branching, and conformation (Ohno et al, 1995). Three conformations of glucans have been reported, the triple-helix, the single helix and a random coil (Yadomae et al, 1996). The structure activity relationships (SAR) of glucans are not well defined. Several studies have suggested that the triple-helix is the most active conformation (Kojima et al, 1986; Yanaki et al, 1983), however, more recent reports suggest that the single helix is the active conformer (Saito et. al., 1991; Aketagawa et. al., 1993). While it is likely that both conformations have biological activity, distinguishing the differences in activity is complicated by difficulties in preparing different stable conformations of a single type of glucan for comparative SAR studies.

Previous studies have reported that the dye aniline blue contains an impurity, sirofluor (sodium carbonylbis(4-(phenyleneamino) benzenesulfonate), that binds specifically to 1→3-β-Glucans (Nicholas et al, 1994; Evans et al, 1984; Smith and McCully, 1978; Faulkner et al, 1973; and Thistlethwaite et al, 1986). When bound to 1→3-β-glucans, sirofluor fluoresces (ca. 140 times increase). The marked enhancement of fluorescence induced by binding to 1→3-β-glucans is thought to occur via H-bonding rather than hydrophobic bonding . We have used this property of aniline blue to study the conformation of SPG, a non-ionic (1→6) branched (1→3)-β-D-glucan polysaccharide, which normally exists as a rigid rod-like triple helix in aqueous solution and which has antitumor activity and good water solubility (Kojima et al, 1986). The objectives of this study were to evaluate the interaction between aniline blue and SPG after treatment with NaOH, to stabilize NaOH treated and neutralized SPG in different conformation ratios (single helix:triple helix), and to evaluate the structure-activity relationship of the different conformations of SPG by reacting with a glucan responsive *Limulus* amebocyte lysate assay (LAL).

Materials and Methods

Chemicals

Laminarin and lipopolysacchride (LPS) were obtained from Sigma(MO, USA) and schizophyllan (SPG) was provided by Dr. David L. Williams of the East Tennessee State University. SPG was obtained in sterile water (10 mg/mL). Aniline blue was purchased from Polysciences Inc. (Warrington, PA). Limulus amebocyte lysate (LAL) reagents were purchased from Associates of Cape Cod (ACC, Pyrotell-T)(Woods Hole, MA) and Whittaker Bioproducts (WB)(Walkersville, MD).

Steady-State Fluorescence Measurements

Steady-state fluorescence measurements were carried out on a SLM 8000C or ISS photon-counting spectrofluorometer. Spectral measurements were made at 395 nm excitation, and emission (maximum at 500 nm) spectra were corrected for solvent background and Raman scattering. To avoid the inner filter effect, the absorbance of solution was controlled below 0.05. Temperature was controlled at 20 ± 0.1 °C unless otherwise mentioned. The slow kinetic fluorescence intensity was measured on ISS instrument while constantly stirring. The rate of aniline blue bound with glucans was calculated by a non-linear curve fitting equation.

Denature-Renature Process of Glucans

At NaOH concentration above 0.25M (pH ~ 13), glucans are reported to have a random coil conformation (Kitamura et al, 1996) and to stain with Aniline blue (Nagi et al, 1993). SPG and laminarian were treated with NaOH to pH 13, adjusted to pH 11.5 and 7 (neutralized), respectively, after which aniline blue was added to the solution. The experimental procedure for SPG fluorescence denature-renaturation was as follows: 25 uL of SPG was added to 1 mL 0.25M NaOH (in 0.5 M KCl); after 20 min. the pH was adjusted to about 11.6 by adding 1N HCl. After 2 minutes

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this solution was diluted with 10 mL pH 11.5 Na_2HPO_4-NaOH buffer (in 0.5 M NaCl).

For binding with aniline blue, 3 uL 5.52×10^{-4} M (0.436 mg/mL) of aniline blue was added to 1 ml of the glucan preparation at different times after neutralization and the fluorescence of the preparations evaluated.

LAL Assays

A glucan responsive kinetic turbidimetric *Limulus* lysate from Associates of Cape Cod was used to evaluate the biological activity of glucan (Remillard et al, 1993). Renatured glucan solutions were dialyzed against pyrogen free distilled water for 1 day before the LAL assay was performed. LAL assays were performed in flat-bottomed 96-well microtiter plates as follows. Eighty microliters of glucan samples, pyrogen free water, or endotoxin standards were placed in microtiter plate wells and 20 ul of pyrotell T (Associates of Cape Cod) added to each well. The plates were incubated at 37 °C and the absorbance of each well was measured every 30 second at 340 nm for 40 min., using a microplate reader (Kinetic-QCL, Whittaker Bioproducts) and a communication software (Anthos reader V. 2). The onset time, define as a change of optical density (ΔOD) of 20 mOD, was used to prepare the standard endotoxin curve. The range of the standard curve was from 0.0005 to 50 LPS ng/mL. The reactivity of glucans was converted to the corresponding LPS concentration. A LPS 5 ng/mL and sterile pyrogen free water were used as the positive and negative controls.

To determine if the glucan preparations were contaminated with endotoxin another LAL assay was performed using a lysate (WB) that is rendered insensitive to glucans. Serial 10-fold dilutions of a 50 ng/ml LPS solution were used to prepare an LPS standard curve. 50 ul of the LAL reagent water blank, LPS or glucan samples were put into the appropriate wells of a 96-well microplate. Plates were pre-incubated for 10 minutes after which 50 ul of LAL was added to each well. The absorbance of each plate at 340 nm was measured continuously using a microplate reader (Kinetic--QCL, Whittaker Bioproducts) and the reaction ended when the onset time, define as a change of optical density (ΔOD) of 200 mOD, for each well was reached.

Results and Discussion

We have investigated several factors which affect the fluorescence intensity of glucan-aniline blue complexes. These include: ionic strength, buffer pH, NaOH concentration used for denaturation, and the effect of temperature on aniline blue staining time.

Effect of Ionic Strength on Fluorescence Intensity

Ionic strength was found to enhance fluorescence of both NaOH treated and untreated aniline blue-laminarin complexes. However, the untreated triple-helix conformer of SPG did not bind with aniline blue. When SPG was treated with NaOH, neutralized and bound with aniline blue, changes in ionic strength did enhance the fluorescence intensity. These data indicate that for studies comparing the activity of different glucans treated with NaOH the ionic strength must be controlled and suggest that aniline blue binds more easily with single helix conformers. For comparisons in this study all treatments were done at near saturation (0.5 M KCl) to avoid the effects of ionic strength.

Effect of Buffer Solution's pH and NaOH Denaturing Concentration on Fluorescence Intensity

Aniline blue fluorescence only when complexed with glucans. Evans et al. (Evans et al, 1984) demonstrated that laminarin-aniline blue fluorophor undergoes an increase in fluorescence intensity as the pH is increased from 3 to a maximum at about 11.5 after which fluorescence drops to zero at pH levels higher than 13. We observed similar results over a pH range of 10.5-13 using a different pH buffer system. In neutral solution, SPG does not complex with aniline blue to induce fluorescence intensity. Even in pH 11.5 buffer, where aniline blue-lamanarian complexes showed maximum fluorescence sensitivity , only a very low amount of fluorescence intensity was observed. These data support the observation that the triple helix of SPG does not readily complex with aniline blue. However, after denaturing SPG with NaOH (≥0.25M) then neutralizing and complexing with aniline blue, SPG also showed a peak fluorescence intensity at pH=11.5 similar to that observed for laminarian. Furthermore, we observed that SPG preparations denatured with high concentrations of NaOH had a higher fluorescence intensity after neutralization and binding with aniline blue than those denatured with lower concentrations. These data suggest that higher concentrations of NaOH increase the proportion of single-helix conformers of SPG.

Effects of Staining Time and Temperature on Fluorescence Intensity

The time required for SPG and laminarin aniline blue complexes to develop their maximum fluorescence intensity at 20 °C were 3.5 and 1 hrs, respectively. If the temperature was increase to 25°C, the staining for maximum fluorescence intensity for SPG occurred within 30 minutes. This decrease in staining time may be related to the increase in thermal energy which increases the molecular motion of the molecule resulting in a more rapid binding with aniline blue. For conformation comparison studies we used a constant temperature and staining time for all preparations.

Slow Kinetics of Fluorescence after Addition of Aniline Blue to Renatured SPG Over Time

The kinetics of fluorescence in adding aniline blue on consecutive days following renaturation of SPG are shown in (Figure 1). For all but day 5, the relative height of fluorescence intensity at a fixed time (for example, 4500 sec.) decreased with the increasing number of days. The reason the fluorescence intensity at day 5 is less than day 3 or day 4 is unclear at this time. One possibility may be that

the solution used on that day was not homogenous and resulted in a discontinuity in the dye-glucan binding complex. Alternatively, there may be little change in the molecular conformations of the renatured glucan over days 3-5 and the development of fluorescence will be similar and indistinguishable using this technique.

The small difference in fluorescence at days 3-5 is confirmed by Figure 2 which shows the fluorescence emission intensity of aniline blue added to renatured SPG at different days. There was a decreased in fluorescence intensity with increasing number of days, which corresponds to the decrease in single helix conformers in the preparation.

The reaction between aniline blue and glucan appears to be irreversible at 20°C. If the staining reaction were reversible, we would expect to observe a change in fluorescence intensity as the glucan renatures, however, after addition of the dye the fluorescence intensity remained constant for up to 15 days.

Response of a Glucan Specific LAL to Different Ratios of Single:Triple Helix SPG Conformers

We evaluated the different conformations ratios of aniline blue treated SPG to an LAL from ACC that is responsive to both endotoxins and glucans. Background endotoxin levels of the preparations were evaluated using an LAL that responds only to endotoxin (WB). The activation of different concentrations and different conformations of SPG on of two LALs are shown in Table 1. There was only a limited response of each SPG preparation (t= 0-105 hours) to the LAL from WB, indicating there was limited contamination from endotoxin in the preparations.

Conformations of SPG showing the highest reactivity to the glucan responsive LAL contained the highest proportion of single helix conformers (t=0). The day-to-day decrease in fluorescence intensity in SPG-aniline blue system was correlated with the decrease in LAL activity (Figure 2 and Table 1). These data demonstrated that one type of biological activity (LAL enzyme activation) was correlated with conformational change found in the fluorescence spectrum. Nagi et al. used the G test to evaluate the activity of NaOH treated and neutralized SPG at day 1 and day 7 (Nagi, et al., 1993). They suggested that about 77% of the single helix conformer would revert to the triple helix over seven days. We observed the same trend in these experiments. In Figure 1, the fluorescent intensity decreased from day 2 to day 7 by 39% and LAL activity decreased by 85% from day 0 to day 4 (105 hours) (Table 1).

Conclusions

NaOH treatment of triple helix glucans will result in the formation of single helix conformers. After neutralization of NaOH treated triple helix glucans, the single helix conformers will gradually revert to the triple helix conformer. Only single helix forms of glucan bind with aniline blue. Differences in ionic strength were found to enhance fluorescence intensity of aniline blue-single helix glucan complexes. The rate of glucan-aniline blue complex formation was dependent on temperature and the concentration of NaOH used to denature triple helix glucans. The reaction between aniline blue and glucan appears to be stable as shown by the constant fluorescence intensity of glucan-aniline blue complexes. Addition of aniline blue at different times after neutralizing NaOH treated triple helix glucan will produce different but stable ratios of single helix to triple helix glucan conformers. Glucan conformers with a higher proportion of single helix conformers were more reactive with a glucan sensitive LAL than glucans with a higher proportion of triple helix conformers.

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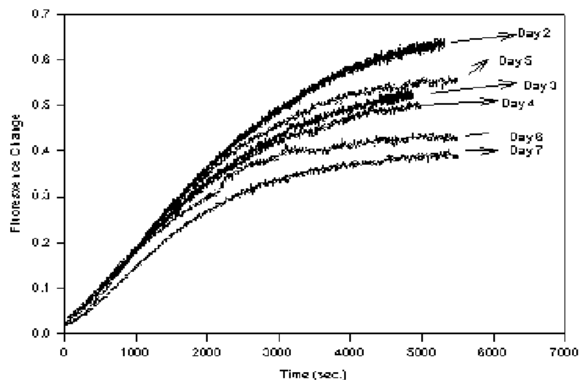

Figure 1. Fluorescence intensity of Schizophyllan after adding aniline blue on successive days after neutralization.

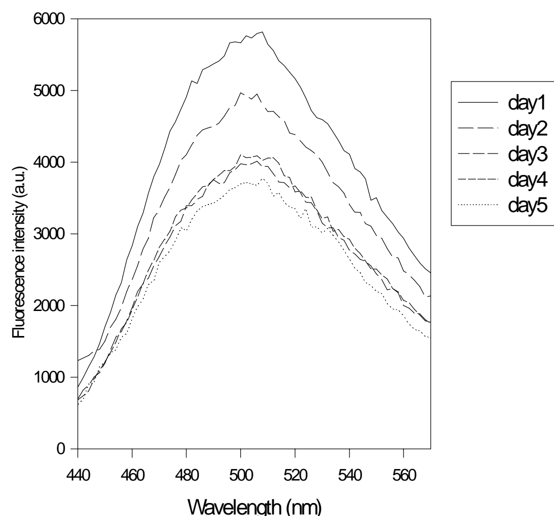

Figure 2. The decrease in fluorescence intensity of renatured SPG from days 1-5.

Table 1. Effect of different conformations of SPG stabilized with aniline blue after denaturation (SPG, 9.23 ug/mL pH=7.8)

Aniline blue addition (hrs)	0	17	32	43	60	73	105
Corresponding LPS ng/mL (from WB)	0.34	0.53	0.39	0.26	0.20	0.13	0.02
Corresponding LPS ng/mL (from ACC)	68.8	58.3	46.0	39.0	34.4	29.6	10.6