OBSERVATION OF A PARTIALLY-OPENED TRIPLE-HELIX CONFORMATION IN 1¬3-β-GLUCAN BY FLUORESCENCE RESONANCE ENERGY TRANSFER SPECTROSCOPY

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Abstract

Molecular conformation is one of the important factors in understanding the structure-activity relationship of 1→3-β-glucan. However, a direct observation of glucan conformation in solution is very difficult. Therefore, the fluorescence resonance energy transfer (FRET) spectroscopy was applied as an indirect method to study the conformational change of glucans induced by NaOH. Three conformations of glucan have been reported in solution: triple-helix, single helix, and random coil. Previous studies have suggested that treatment of the triple-helix conformation with NaOH produces single helix conformers. We propose that a partially-opened triple-helix rather than a single helix, is formed by treating the triple-helix glucan, laminarin with NaOH. Fluorescence resonance energy transfer (FRET) spectra of the glucan, laminarin, doubly labeled with 1-aminopyrene (AP) as donor probe and fluorescein-5-isothiocyanate (FITC) as acceptor probe attached at the reducing end, showed that a partially-opened triple-helix conformer was formed on treatment with NaOH. The conversion between the triple-helix and partially-opened conformation was reversible (when denaturation was mild, [NaOH] < 1 M). Different degrees of conformer opening could be stabilized by adding aniline blue, which binds to the single helix portion the partially-opened triple-helix glucan. Different conformations were stabilized with aniline blue when examined using the *Limulus* amoebocyte lysate (LAL). The results show that conformations stabilized at different times activate the LAL assay differently, thus demonstrating that the biological activity was dependent on the degree of strand opening.

Introduction

Three conformers of soluble glucans have been reported, including: a single helix, triple-helix and random coil. The conversion between triple-helix and single helix can be mediated by different chemical or physical treatments [1]. For example, treatment of the triple-helix, SPG with NaOH followed by neutralization and dialysis against distilled water is a method for preparing single helix–rich forms of SPG from triple-helix conformation [2,3]. Over time, the single helix will revert to the triple-helix, which is the more stable form [2]. Aketagawa et al. suggested that the mechanism of NaOH treatment involved a conformational change from the triple-helix to single chains which rapidly converts to the single helix, followed by gradual reversion to the triple-helix [4]. This mechanism implies that, immediately after denaturation, the MW of NaOH-treated glucans should be one third of that of the native glucans. However, this was not supported by experimental evidence, which indicated that denatured SPG (treated with NaOH) only gradually reverts to the triple-helix [2], and native SPG has the same MW as denatured SPG [5, 6]. An alternative explanation for the SPG experimental results is that a partially-opened triple-helix may be formed in the NaOH-induced denature-renature process. To further understand the structure - activity relationship of glucan, we hypothesize that the biological activity of glucans depends on the degree of strand opening. We use FRET, combined with *Limulus* amebocyte lysate (LAL), to evaluate the relationship between conformation and one type of biological activity, LAL reactivity.

Materials and Methods

Triple-Helix Conformation of Laminarin

This study used laminarin as a example of triple-helix 1→3-β-glucans conformation. The assumption that laminarin has a triple-helix conformation was based on the high-resolution solid-state ^{13}C NMR and laminaran's characteristic X-ray powder-diffraction patterns reported by Saito et al (7,8,9). Laminarin is commercial available and has good water solubility. No extra solvent was needed which avoid the possible solvent effect on the conformation of glucans. This experiment was repeated by using a higher molecular weight 1→3-β-glucans, actigum, to verify the general applicability of this method to triple-helix conformation of 1→3-β-glucans.

Preparation of Laminarin-AP Derivative

The AP probes was designed to specifically label the reducing end of glucans by a modified method after Evangelista et al. [10] as follows: laminarin 100 mg (0.005 m mole) in a 5-mL glass vial was mixed with 2.6 mg (0.011 m mole) AP (in 0.5 mL Me_2SO), 2 mL of 15 % acetic acid, 17.6 mg (0.28 m mole) sodium cyanoborohydride, and 42 mg (0.31 m mole) ammonia sulfate $(NH_4)_2SO_4$. The reaction vial was heated in a water bath at 75°C for 1 h, then dialyzed with double distilled water for 2 days. The final solution was divided into two equal portions; one was reserved as the donor-labeled sample and the other was further labeled with FITC (laminarin-AP-FITC).

Preparation of Laminarin-FITC Derivative

FITC reacts with primary and secondary amines, but not with the hydroxy group of glucans. The laminarin-FITC

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(acceptor only) derivative was prepared by reacting FITC with laminarin-NH_2. The procedure for reductive amination of laminarin (laminarin-NH_2) followed the method of Liu et al. [11]. Laminarin (50 mg) was dissolved in water and placed in a screw-cap glass tube. An excess of 2.0 M of $(NH_4)_2SO_4$ and 0.4 M of $NaBH_3CN$ was added, mixed well, and then kept at 100 °C for 120 min. After completion of the reaction, the solutions were rapidly cooled by putting the tube into an ice bath. After cooling, they were dialyzed against double distilled water 3 times for 2 days. This solution was further derivatized with FITC for preparation of laminarin-FITC: One milliliter of pH 10 boric acid-potassium hydroxide buffer and an excess of FITC (250 uL, 0.006 m mole) were added to the laminarin-NH_2 (0.76 mL) solution at room temperature. The final pH was adjusted to 10 by adding 0.1 M NaOH, continuous stirring for 24 h, and then dialyzing against double distilled water for 2 days to eliminate free FITC and other small molecules.

Preparation of Laminarin-AP-FITC (Donor and Acceptor) Derivatives

Two milliliter pH 10 boric acid-potassium hydroxide buffer and an excess of FITC (250 uL, 0.006 m mole) were added to laminarin-AP solution (1.85 mL) at room temperature. The final pH was adjusted to 10 by adding 0.1 M of NaOH and continuous stirred for 24 h. Then, the solution was dialyzed against distilled water for 2 days. To ensure that all three solutions (donor labeled, acceptor labeled, and doubly labeled) contain the same concentration of glucan, they were adjusted to the same volume.

The labeling ratio of the FITC was estimated from UV absorbance by using the molar extinction coefficient for FITC, ε=73000 @ 494 nm [12]. The labeling ratio of FITC in laminarin was estimated to be about 1 %. The labeling ratio for AP was calculated to be about 10 %, by using a determinated value ε=2618 @ 370 nm. However, actual value for AP labeling maybe slightly smaller than 10 %, due to FITC could replace some of the AP during the derivatization process.

Preparation of the Glucan Solution

Preparation of the glucan solutions for the LAL assays was as follows: 400 uL (1 M) NaOH was mixed with 400 uL laminarin-AP-FITC (~8 mg/mL). The solution samples were neutralized with 1 M of HCl and 10 mL of pH 7 buffer was added to this solution. The samples were equally divided into 10 aliquots for subsequent addition of 6 uL of aniline blue (4.221 mg/mL, with stirring for 1 h) at different times. The main purpose of using aniline blue was to stabilize the conformational change of triple-helix at different degrees of partially-opened three-strand during the renaturing process.

Results

The FRET in Laminarin-AP-FITC

The phenomenon of non-radioactive energy transfer over relatively long distances may be used to characterize the spatial relationship of donor- and acceptor-labeled chain molecules. If a system contains two fluorophores and the donor has an emission spectrum which overlaps with the absorption spectrum of the acceptor, then the excitation energy of the donor can be transferred to the acceptor over relatively long distances. The efficiency (*E*) of this transfer is given by the following equation:

$$E = \frac{R_0^6}{(R_0^6 + R^6)} \quad (1)$$

where R is the distance between centers of donor and acceptor chromophores and R_0 is the Förster critical distance at which 50 % of the excitation energy is transferred to the acceptor (50 % transfer efficiency). R_0^6 is proportional to the overlap integral of the donor emission and the acceptor absorption spectra. The FRET can determine the molecular distances in the range 10~80 Å through measurements of the efficiency of energy transfer between a donor and an acceptor located at two specific sites [13]. In this experiment, the relative fluorescence intensity of donor emission and acceptor emission were considered as an indicator of the extent of FRET.

Figure 1 shows that the UV absorption and fluorescence emission spectra of singly labeled laminarin-AP and singly labeled laminarin-FITC. Laminarin-AP has a maximum UV absorbance at 370 nm and a fluorescence emission maximum at 450 nm. Laminarin-FITC has an UV absorbance maximum at 460 nm, which overlaps with AP emission, and a fluorescence emission maximum at 520 nm. When exciting this system with 370 nm light, normally the 450 nm will be the major emission peak observed. However, if FRET occurs, then a second emission at 520 nm will increase, which corresponds to energy transfer from AP to FITC. The spectral overlap between donor emission and acceptor absorption results in an R_0 value determined to be 23.7 Å. This R_0 value provides a maximum measurable distance of 42.6 Å (a range of 1.8 R_0) for FRET in this system.

Figure 2 compares the fluorescence spectrum of single and doubly labeled laminarin. If FRET occurs, an increase in 520 nm peak intensity (or decrease in 450 nm peak intensity) will be observed when two strands come near each other, and a decrease in 520 nm peak intensity (or increase in 450 nm peak intensity) when they are further apart. The resulting fluorescence spectrum indicates the distance between the donor and the acceptor probes. Because the laminarin-AP-FITC was derived from laminarin-AP and the concentration of both was equal (material and methods section), the peak intensity of AP should be approximately the same for laminarin-AP and laminarin-AP-FITC if no energy transfer occurs. However, we observed that the peak intensity of AP in laminarin-AP-FITC was 3-fold lower than the precursor laminarin-AP, indicating that some degree of FRET had occurred in the

laminarin-AP-FITC molecule. About a two-fold increase of the AP peak was observed after treatment of the triple-helix with different concentrations of NaOH, which causes the three strands to denature (Figure 3). Treating glucan with NaOH at a concentration greater than 0.24 M was assumed to be the requirement for the triple-helix glucan to became the random coil [6,14]. This random coil is reported to be converted to the single helix form after removing NaOH [1]. If this is a true strand separation, the FRET will disappear due to the relatively large distance between the donor and the acceptor probe, and the AP intensity of laminarin-AP-FITC should return to about the same height of laminarin-AP. However, the peak increased to only 2/3 of the height of laminarin-AP (compare Figures 2 & 3). In Figure 3, the same three concentrations of laminarin-AP-FITC underwent the same denature-renature process but at different NaOH concentrations. The highest concentrations of NaOH gave the most intense donor emission and also the lowest acceptor emission. This suggests that the increasing concentrations of NaOH cause increasing degrees of three-strand opening. To rule out the possibility that the FRET results from inter-molecular interaction, we measured the fluorescence spectra after mixing the singly-labeled laminarin-AP and laminarin-FITC. No significant FRET was observed (Figure 4). Because the inter-molecular distance in the studied system is large, FRET is most likely observed only from donor and acceptor probes of the same glucan molecule. Therefore, the spectrum of laminarin-AP plus laminarin-FITC in a solution represents background spectra without FRET.

Figure 5 shows the fluorescence emission spectrum over time of a denature-renature laminarin-AP-FITC sample with NaOH as the denaturing agent. At 0 h, the fluorescence intensity was highest at 450 nm and lowest at 520 nm, indicating that the donor and acceptor probes were far apart. As the time increased, the 450 nm peak decreased, whereas the 520 nm peak increased, indicating that donor and acceptor were approaching each other. Complete conversion took about 8 days, at which time most of the fluorescence emission came from the acceptor. This indicates that the donor and acceptor were close to each other. The concentration of NaOH used in this experiment was lower (0.0115 M) than the concentration (0.24 M) previously reported necessary to convert the triple helix to random coil [6,14]. The time dependent FRET phenomenon was observed in all concentrations of NaOH used (from 0.0115 M to 0.833 M, Figures 3&5).

Stabilized the Conformational Change by Adding Aniline Blue at Different Times

The conformation of laminarin-AP-FITC as it renatures can be stabilized by adding aniline blue at different times. In Figure 6, each spectrum represents the same concentration of laminarin-AP-FITC, but with aniline blue added at different times. For each preparation staining with aniline blue was completed within one hour. The relative difference in peak intensity at 450 and 520 nm indicate that the triple-helix strands of the stained glucans are in different degrees of opening.

Structure-Activity Relationship

Reactivities of different conformations stabilized by adding aniline blue at different times toward the LAL assay. The same solutions that were stained by aniline blue were evaluated after 20 days with the LAL assay. The FRET spectrum in Figure 6 was taken on the 20th day after denaturation and addition of aniline blue, indicating that aniline blue stabilized the preparations which were used for the LAL assays. The highest intensity at 450 nm corresponds to aniline blue added at 11.5 h after renaturing. This conformation corresponds to the largest separation between donor and acceptor. The LAL activity, as indicated by onset time, decreased with increasing time after the addition of aniline blue (Figure 7).

For the LAL assay, the time required for the development of turbidity is inversely proportional to the solution concentration [15]. Because the concentrations of glucan used in fluorescence measurement in Figure 6 were the same, the different LAL onset times are related to the different conformations of glucan. Comparison of the data in Figure 6 and Figure 7 shows that a 5-fold decrease in emission intensity of the donor probe at 450 nm (Fig. 6) correlated to an increase of ~40 % onset time in LAL assays (Fig. 7).

Discussion

The design of the FRET system was to tag the donor and acceptor fluorophore groups at the reducing end of glucan strand; therefore, the inter-hydrogen binding of the triple-helix glucan was not altered. This study provides evidence that NaOH-treated glucan exists as a partially-opened triple-helix rather than single helix form as suggested by Aketagawa, et al.[4]. Our conclusion is based on the following evidence: In order for FRET to occur, the donor and acceptor must be in close proximity. If NaOH treatment produces single helixes, then FRET should be observed after denaturing the mixed AP and FITC labeled glucans. However, no significant FRET was observed in this system, indicating that dissociation and re-association is not occurring after the denaturation of glucans (Figure 4). Additionally, samples of laminarin-AP-FITC treated with different concentrations of NaOH above 0.24 M had a different intensity at 450 nm (Figure 3). If complete dissociation occurs after NaOH treatment, we would expect that there will be no difference in intensity of the different preparation at 450 nm.

We propose that the mechanism of conformational change by NaOH treatment is via a partially-opened triple-helix as shown in Figure 8. The triple-helix conformer of glucan is

formed by three hydrogen bonds (H-bonding) to oxygen in the C-2 position [16,17]. Treatment with NaOH can break these H-bonding resulting in a partially-opened triple-helix structure. The degree of strand opening depends on the concentration of NaOH used. This structure can better explain the constant MW observed in native and denatured SPG [5,6]. However this partially-opened triple-helix is difficult to identify, since most spectroscopy methods can not distinguish between a close triple-helix and a partially-opened triple-helix. By using FRET, we can monitor the degree of strand opening in different concentrations of NaOH. Although this structure has not been previously proposed as the intermediate for NaOH treatment, electron micrographs in a recent study of SPG have shown partial strand opening with three distinct single strands [18].

In derivatizing the laminarin we sought to avoid modifying the native conformation of the close triple-helix form, however, after derivatization the triple-helix glucan has some degree of partially-opened structure in the end groups (laminarin-AP-FITC in pH 7 buffer, Figure 2). Therefore, this conformation is not the same as the native triple-helix (the final conformation in Figure 5). However, we still can observe the dependence of NaOH concentration on the strand opening and the dynamic conformational change behavior during the renaturation process (Figures 3&5). The renaturing process can be interrupted by adding excess aniline blue (Figure 6). Contrary to previous reports [6, 14], which indicate that 0.24 M NaOH is required to alter glucan conformation, we observed strand-opening effects at all NaOH concentrations tested over a range 0.0115 to 0.83 M.

For these experiments we can not rule out that treatment of triple-helix β-(1→3)-D-glucans with NaOH may also cause untwisting at the middle of the helix. Since we labeled the ends of the glucan strand with probes, donor-acceptor distances would not be affected by untwisting at the middle of the helix. Therefore, using the labeling method at the reducing end can not detect the conformational change of those glucans untwisting from the middle.

The single helix conformation of glucan is thought to be the form that activates the LAL assay [5]. We speculate that the activity of glucan is also related to the partially-opened triple-helix conformation. If this speculation is correct, stabilization of this conformation may affect the biological activity of glucan. We used aniline blue to stabilize the conformational change in renaturing glucan (Figure 6). The differences in conformation were confirmed by FRET. When these were evaluated using the LAL assay, we showed that the conformational changes correlated well with the LAL activity. The relationship between the conformation and LAL activity was examined for laminarin and actigum (Figure 7).

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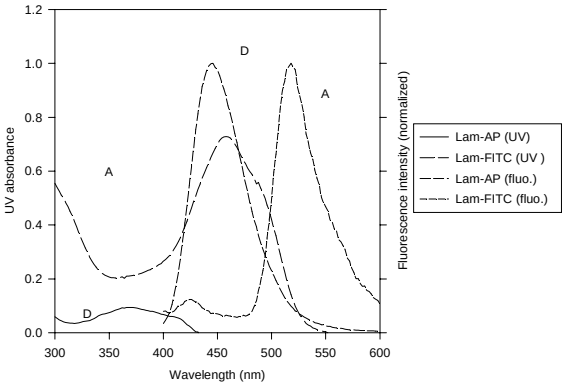

Figure 1. UV & fluorescence spectra of laminarin-donor and laminarin-acceptor derivatives (The fluorescence intensity has been normalized; A the acceptor (FITC) and D is the donor (AP) fluorophor).

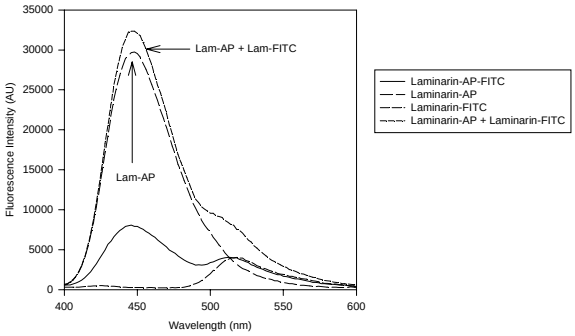

Figure 2. Fluorescence spectrum of laminarin-AP, laminarin-FITC, and laminarin-AP-FITC. Twenty uL of laminarin-AP, laminarin-FITC, and laminarin-AP-FITC (~8 mg/mL each) were added to pH 7 buffer as indicated in legend, for the same total of 820 uL in solution and fluorescence spectrum was measured immediately.

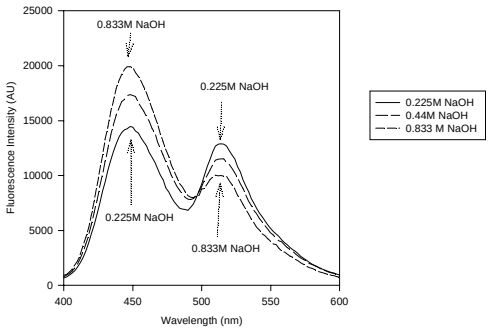

Figure 3. The effects of NaOH concentrations in laminarin-AP-FITC fluorescence spectrums. Laminarin-AP-FITC (~ 8 mg/mL) 20 uL was denatured with 0.27 M, 0.5 M, and 1 M of NaOH, 1N of HCl was added to neutralize the solution. The final solution volume was adjusted to 820 uL with pH 7 buffer.

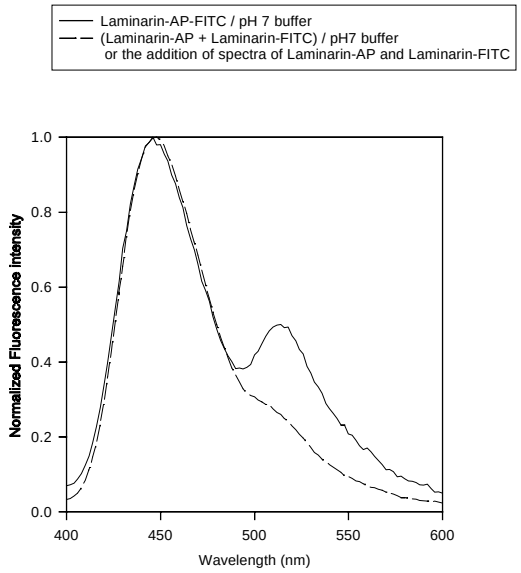

Figure 4. Comparison of the fluorescence spectrum between the sum of the singly labeled to doubly labeled laminarin (normalized at 450 nm).

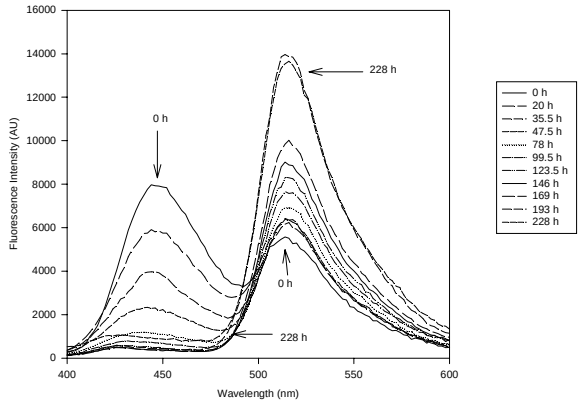

Figure 5. The conformational change of renaturing laminarin-AP-FITC detected by FRET. Laminarin-AP-FITC (~ 8 mg/mL) 40 uL was denatured with 0.016 M of NaOH, neutralized with HCl, added pH 7 buffer, measured at different hours.

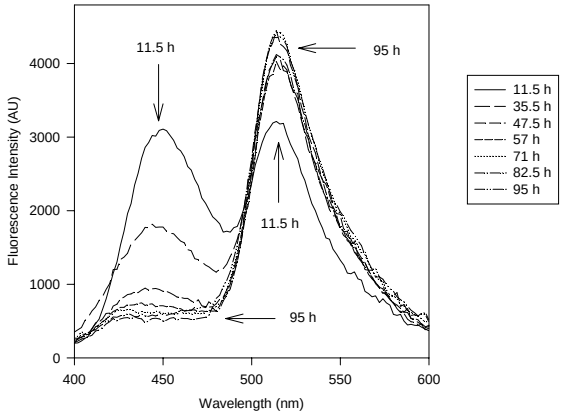

Figure 6. Adding aniline blue at different time stabilized the Laminarin-AP-FITC conformation at that time. (0.5 M of NaOH was used as the denaturing solution, neutralized with HCl, diluted in pH 7 buffer, measured after 20 days).

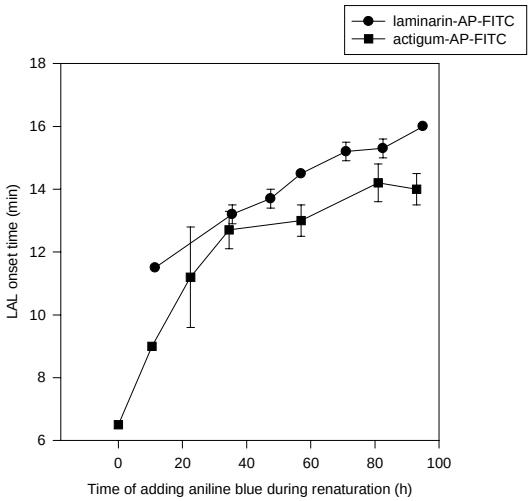

Figure 7. LAL activity of glucan-AP-FITC vs. renaturing glucan added aniline blue at different hours.

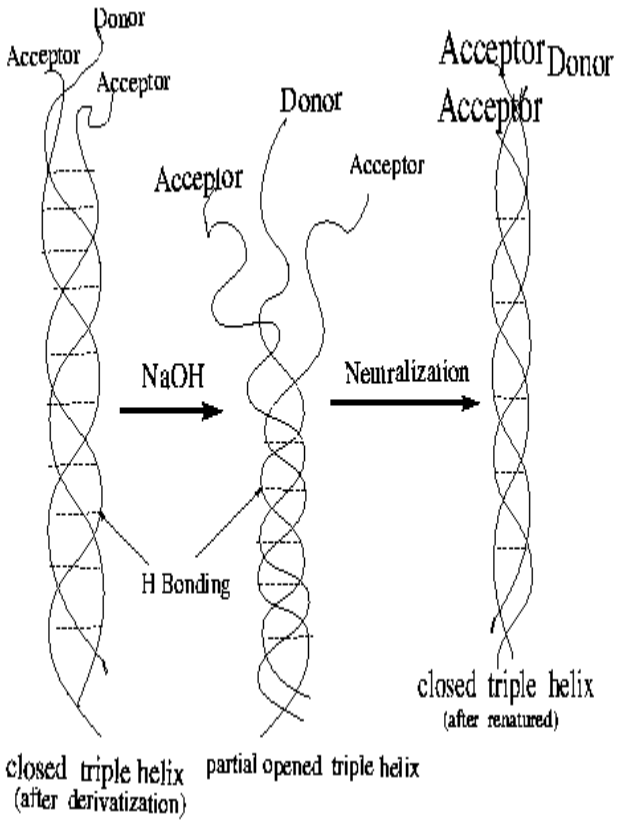

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Figure 8. The proposed mechanism of conformational change involving partially-opened triple-helix.