Alternation of Two Forms of Restriction Endonuclease HindIII

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Two forms of the restriction enzyme HindIII were alternated with each other under some physiological or biochemical conditions. Addition of a low amount of phase T7 to the culture of HindIII-producing Haemophilus influenzae Rd, resulted in appearance of some amounts of the P2 fraction of HindIII, which was eluted with a high concentration of KCl from a phosphocellulose column. Higher amounts of T7 caused a decrease of the P2 fraction; finally the alternative P1 fraction of HindIII, which was eluted with a lower concentration of KCl, remained exclusively.

Addition of disaccharides such as maltose and trehalose to the bacterial extract, yielded more P2, although the disaccharides inhibited to this enzyme. Urea showed an interesting distribution of these two forms of HindIII. Phosphocellulose chromatography in the presence of 2 M urea generated a broad peak of HindIII Activity. Addition of 4 M urea, on the contrary, showed only one active peak of this enzyme. The HindIII could be purified by the following DEAE-cellulose chromatography.

These results indicate the presence of only one kind of HindIII molecule, which was alternated between free and bound forms, and a certain kind of factor that would equilibrate these two forms.

Key words: HindIII; maltose; phage T7; renaturation; restriction enzyme

Many kinds of restriction endonucleases, especially type II enzymes, which recognize and catalyze palindromic sequences of DNA, have been discovered and characterized. It has been regarded that microorganisms can survive by producing restriction endonuclease against viral infection. The DNA of the microorganism itself is methylated specifically by the cognate methyltransferase, to avoid breakdown by the endogenous restriction enzyme. However, the mechanisms by which they regulate their restriction endonucleases and methyltransferases, are still unclear. For example, the viral DNAs are never methylated by the methylase before the attack of restriction enzymes. There might be some factors which mediate these enzyme activities or their genetic expression.

Gene structures of some of them are known, showing that a restriction endonuclease gene is always adjacent to the gene for its cognate methylase. Brooks et al. reported that there is an open reading frame between these genes for BamHI. The protein coded by this frame and expressed artificially, acted as an activator of BamHI, while no such activators were actually present in the BamHI-producing bacteria, Bacillus amyloliquefaciens.

In a previous paper, we reported the presence of two forms of the restriction enzyme HindIII in the extract of Haemophilus influenzae Rd. In phosphocellulose chromatography, the P1 and P2 fractions of HindIII were eluted with low and high KCl concentrations, respectively. The HindIII could be purified with ease from the P1 fraction by DEAE-cellulose chromatography. However, the HindIII in P2 fraction was still contaminated with other proteins in DEAE-cellulose chromatography, and finally purified by DNA-cellulose chromatography. The P2 fraction disappeared when high M.O.I. (the ratio of phage to the bacteria) of phage T4 or T7 was added to the bacterial culture.

This paper describes the effects of T7 on alternation of HindIII. Phage T7 with low M.O.I. caused accumulation of P2, while the P2 decreased when a high M.O.I. of T7 was used. We also report the biochemical alternation between two forms of HindIII. Addition of disaccharides such as maltose and trehalose to the extract caused a change of distribution of P1 and P2. Addition of 4 M urea generated a single peak of HindIII activity, suggesting that there is only one molecular species of this restriction enzyme.

Materials and Methods

Haemophilus influenzae Rd, which produces HindIII, was a gift from Nippon Gene. Control restriction enzymes EcoRI and HindIII were purchased from Nippon Gene. Ion-exchange resins, phosphocellulose P11 and DEAE-cellulose DE53, were obtained from Whatman. T7 lysozyme was a laboratory stock. Trehalose, maltose, and urea were purchased from Wako Pure Chemicals.

HindIII activity assay: As described in our previous paper, a plasmid, pHA47, which consists of the plu III vector (3.4 kb) and cDNA for human aldolase A, was used as a substrate. The aldolase A cDNA (1175 bp) was inserted at EcoRI and HindIII sites of the vector. The reaction mixture containing 10 mM Tris HCl, pH 7.5, 10 mM MgCl2, 1 mM DTT, and 50 mM NaCl, was added to a test sample and the plasmid (42 ng), which had been treated previously with EcoRI. The reaction was done at 37 C, and the DNA fragments obtained by EcoRI and HindIII digestions were separated by 1% agarose gel electrophoresis and stained with 0.5 μg/ml ethidium bromide. The arrows in Figs. 1, 3, and 4 indicate the desired product (1175 bp) of EcoRI-HindIII digestion. The arrow in Fig. 2 indicates the 3.4 kb product, because inhibitors made the band of 1175 bp faint. Commercial HindIII (0.2 units) was used as an authentic sample, being designated as C in Figs. 1 through 5.

Purification of HindIII. In every study, Haemophilus influenzae Rd was cultured at 37 C in 0.3 liter of Brain Heart Infusion (Difco) which was mixed with 10 mg liter hemin and 2 mg liter NAD. The bacteria was harvested at their stationary phase. When the effects of T7 were examined, T7 lysate was added to the culture at the stationary phase with the specified M.O.I. When 10 μd of 3 x 1011 pfu ml of T7, for example, was added to 3 x 1011 bacteria, we counted the M.O.I. to be 1.0. The culture was continued for two more hours, and harvested. The cell paste was disrupted in buffer A (10 mM Tris HCl, pH 7.6, 0.1 M NaCl, 1 mM EDTA, 100 μg/ml each of aprotinin, leupeptin, pepstatin, and 5% glycerol) to a protein concentration of 10 mg/ml.
containing 0.1 mM EDTA, 1 mM 2-mercaptoethanol, and 10% glycerol) by a Branson Sonifer cell disruptor 200 for 3 min; the procedure was repeated twice with 1-min intermissions. The sonicated material was centrifuged for 10 min at 27,000 × g. The supernatant was then fractionated with 30 70% saturated ammonium sulfate.

The precipitate with 70% saturated ammonium sulfate was dialyzed against buffer A (this sample was called crude extract), and put onto a phosphocellulose column (1.6 cm × 14.5 cm) equilibrated with buffer A. A gradient of KCl in buffer A (from 0 to 1 M, total 120 ml) was put on this column, and 1-ml fractions were collected. KCl concentration in each eluate was measured by a Conductivity Meter CD-35M II, M&S Instruments Inc. When the effect of disaccharide or urea was examined, the reagent was added to the crude extract and incubated at 37 °C, before putting samples onto the column. These reagents were present throughout the phosphocellulose chromatography. Further purification of HindIII was done by DEAE-cellulose chromatography.

SDS-PAGE was done as described by Laemmli.51

Results and Discussions
Effects of phage T7

The Haemophilus influenzae Rd was not lysed nor killed by any M.O.I. of the virulent phage T7 used in this study. The bacteria probably exerted its restriction-modification system for its survival. However, the wet weight of the cell paste was 1.92 g, a little less than the uninfected control sample (2.10 g); the bacteria were damaged to some extent.

Figure 1 shows phosphocellulose chromatography of samples with or without phage attack. The UV absorption profile of the T7-infected sample was different from the control, as shown in Fig. 1A. It is unchanged, however, when the M.O.I. was decreased. In Fig. 1A, HindIII activities in the fractions of the sample with high M.O.I. of T7 are compared with those of control. In Fig. 1B, the activities in the fractions with the lower values of M.O.I. are illustrated. There appear two fractions with HindIII activity, P1 and P2, in every experiment. However, it was found that the P2 fraction, which was eluted with high KCl concentration, disappeared when the M.O.I. was as high as 1.0. When the M.O.I. was lower, the extract was rich in the P2 fraction. Alternatively, the P1 fraction, which was eluted with lower KCl concentration, increased in proportion to the M.O.I., the ratio of T7 added to the bacterial culture. We calculated the activity ratio of P1 to P2 by the following method. Relative activities of 10 μl of combined active fractions were measured by DNA bands, which were stained with ethidium bromide, on a personal computer, as described by us.51 The relative activity of each fraction was multiplied by the total volume of the active fraction, and the resultant values corresponding to total activity

Fig. 1. Effects of Phage T7 in Phosphocellulose Chromatography of HindIII Extracts.
Phage T7 of M.O.I. 1.0, control (A) and M.O.I. 0.5, 0.1, and 0.02 (B) were added to Haemophilus influenzae Rd at its stationary phase. Absorption profiles at 280 nm are drawn at the bottom in A, solid. + T7, broken. control. KCl concentration was expressed with dotted lines. HindIII activities shown in insets were assayed by reaction of 10-μl samples with 42 ng of pHA447 that had been treated with EcoRI, in a 30-μl cocktail, overnight, and are shown in insets. In (B), activity was assayed for the common number of fractions.
was compared. The activity ratios (P1/P2) are as follows: control, 2; M.O.I. 0.02, 0.3; M.O.I. 0.1, 0.5; M.O.I. 0.5, 1.

We previously reported that the P2 fraction was an active form, bound more specifically to the substrate DNA sequences, and coexisted with certain kinds of substances. The results shown in Fig. 1 imply destruction of the P2 fraction by the attack of phage T7. Appearance of the P2 fraction was rather increased when the M.O.I. of T7 was 0.02; and this fraction gradually disappeared when M.O.I. was raised. The population of the P1 fraction increased alternatively: its content was much less than the control, and gradually recovered with increasing M.O.I. The possible explanation for these phenomena is that a factor is induced when the phage attack was taken place, which is digested as M.O.I. was raised. We think this factor binds to HindIII to form more P2 fraction. This factor would work, accordingly, in a protective machinery against phage as well as in stabilization of the restriction enzyme HindIII.

Although we found that the activity pattern shown in Fig. 1 was unchanged even if the crude extract was left at 4°C for more than a week (no spontaneous transition between P1 and P2 fractions occurred, data not shown), the extract was separated by phosphocellulose chromatography as soon as the dialysis was ended.

We do not have enough of an idea about the bacterial regulatory mechanisms against phage: M.O.I., growth phase, phage dose, or period after phage addition should be checked in detail.

Effects of disaccharides

Uritani et al. described how HindIII had full activity even after vacuum-drying when some disaccharides were present in the drying period. Their interest has been focused on protective effects of disaccharides. We examined their effects on distribution of the two forms of HindIII. Figure 2 illustrates the effects of maltose and trehalose on HindIII activity. Both maltose and trehalose inhibited HindIII. This fact does not suggest contrariety against the protective effect of disaccharide mentioned above. In their study on the vacuum-dried HindIII, the final concentration of disaccharide in the activity assay was quite low so that it could not show inhibition at all.

We treated HindIII extract with 20% maltose at 37°C for 15 min, and did the phosphocellulose chromatography, as illustrated in Fig. 3. The maltose concentration was kept at 20% during the chromatography. Maltose concentration in activity assay was only 3%, which did not hinder HindIII activity. The UV absorption profile of the maltose-treated sample was almost the same as that of the control. The disaccharide has a strong effect on the distribution of the two forms of HindIII: the P2 fraction was increased in the presence of maltose. It could be calculated that about 60% HindIII activity was recovered from the P2 fraction when 20% maltose was used, but the P2 content in the control was 33% in the control (the total activity of P1 or P2 fraction was assayed after dialysis against buffer A where the disaccharide was removed).

We also examined the effects of trehalose on phosphocellulose chromatography. Although trehalose has the most protective effect against vacuum-drying, it showed quite similar effects to maltose in the distribution of the two forms of HindIII (data not shown). Carpenter and

**Fig. 2.** Inhibition of Disaccharides on HindIII Activity.

Plasmid pHAA47 (42 ng) treated previously with EcoRI, was digested at 37°C with 1.2 units of commercial HindIII in 40 μl cocktail for 15 min. Samples of maltose (lanes 2, 3) or trehalose (lanes 5, 6) were added to the final concentrations shown on the top.

**Fig. 3.** Phosphocellulose Chromatography of HindIII Extracts (Effects of Maltose and 2M Urea).

Absorption profile: solid, control and in 20% maltose; broken, in 2M urea. KCl concentration is expressed with dotted lines. HindIII activities are shown in insets. The phosphocellulose chromatography was done in 20% maltose after incubation for 15 min at 37°C in 20% maltose. From each fraction, 5 μl samples were used for activity assay overnight in a total volume of 30 μl. In a case of 2M urea, the chromatography was done in 2M urea after incubation for 15 min at 37°C in 2M urea.
Crowe 8 pointed out that the disaccharides bind to proteins and substitute for water when their hydration shell is removed. The disaccharide probably replaces the substrate water at the active site, and inhibits the enzyme. Also, we consider a new role of the disaccharide as an accelerator of forming complexes in P2 or as its stabilizer. Hydrophobic interaction would be necessary in formation of the complex, and the disaccharide might be helpful in this procedure. Some other organic reagents will be used to examine their effects on alternation of the two forms of restriction enzymes.

Effects of 2 M urea

Urea is a denaturant that absolutely inhibits any enzyme activity. It is likely that high concentrations of urea dissociate homodimeric HindIII to two monomers. Actually we found that HindIII lost 50% of its activity upon exposure to 3 M urea for 5 min at 37°C (data not shown). However, the HindIII could recover its activity by dilution of the urea. We examined the effects of urea on the alternation of the two forms of HindIII. As shown in Fig. 3, phosphocellulose chromatography of HindIII extract in presence of 2 M urea was displayed. Because net charges of proteins were shifted by addition of 2 M urea, the absorption profile at 280 nm of the 2 M urea-treated sample was quite different from those of the control and maltose-treated samples. The urea concentration in the samples for the activity assay was only 0.33 M; we could observe enough activity of HindIII. Surprisingly, the activity spread in many fractions as illustrated in Fig. 3. We consider that HindIII in the P2 fraction is bound to a certain kind of factor(s), while it is free in the P1 fraction. Treatment with 2 M urea yielded denatured forms of HindIII in both P1 and P2 fractions, to which some other proteins might adsorb non-specifically. This would be the reason for the broad peak of activity that emerged after 2 M urea treatment.

Effects of 4 M urea

The crude extract was treated with a higher concentration of urea: it was incubated with 4 M urea for 15 min at 37°C, then put onto the phosphocellulose. This treatment resulted in a completely denatured form of HindIII where no other proteins could bind to it any more. In Fig. 4, the HindIII activity profile forms a single peak around fraction no. 50, differing from those in Figs. 1 and 3. This fact indicates that there is only one molecular species for HindIII. Moreover, HindIII could be renatured even after treatment with 4 M urea (the urea concentration was reduced to 0.67 M in the assay cocktail). We estimate that approximate 80% of the activity of the control (without urea) could be recovered after the phosphocellulose chromatography in 4 M urea and dialysis against buffer A. We think that little irreversible denaturation of HindIII occurred in its exposure to 4 M urea. The active fractions in phosphocellulose chromatography in the presence of 4 M urea were combined, dialyzed against buffer A, and put onto a DEAE-cellulose column (1.4 cm x 9 cm), which was equilibrated with buffer A. A gradient of KCl in buffer A (0-0.2 M, total 50 ml) was put on this column, and 1-ml fractions were collected. Figure 5 illustrates the HindIII activity of eluates. There appeared an active region around fractions no. 15-17. SDS-PAGE of active fractions were illustrated, too. Judging from the results in the previous studies, 3 the lower protein bands around 25 kDa (indicated by the arrows) correspond to the HindIII subunit. Only the active fractions are rich in this
major band. Consequently, the HindIII protein could be purified almost to homogeneity by this procedure.

As mentioned above, we think that a factor that activates HindIII protein is likely to be present in *Haemophilus influenzae* Rd. Some kinds of mammalian proteins such as hemoglobin, RNase, aldolase A, and γ-globulin did not play a role as an activator of HindIII (data to be published). Therefore, there would be a factor which specifically activates HindIII, in *Haemophilus influenzae* Rd. We also found that there were two active forms of EcoRI that were separated by phosphocellulose chromatography (in preparation). Recently, Aggarwal *et al.* reported by X-ray studies at 1.95 Å resolution that the active site of EcoRV is similar to those of BamHI and EcoRI although its overall tertiary structure is different from them. Accordingly, it is possible to imagine the presence of ubiquitous factor(s) that can bind to the common sites of type II restriction enzymes to activate and stabilize them. The factor also mediates an equilibrium between the two forms of the restriction enzyme.

The effects of urea on HindIII are very interesting: 2 M urea yielded a broad activity peak (Fig. 3), and 4 M urea yielded a single peak (Fig. 4). In both cases, HindIII renatured sufficiently. Treatment with 4 M urea generated an isolated form of this enzyme. The enzyme could be purified easily after this treatment. Therefore, urea treatment of crude extracts seems to be quite an efficient method for purification of restriction enzymes.

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References