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Resolution of proteins on a phenyl-Superose HR5/5 column and its application to examining the conformation homogeneity of refolded recombinant staphylococcal nuclease

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Abstract

In order to examine the effect of amino acid substitutions on protein retention in hydrophobic interaction chromatography and the resolution of a phenyl-Superose HR5/5 column, two groups of staphylococcal nucleases, named Y113/W140 (wild-type), Y113W/W140 and Y113/W140F, Y113W/W140F, were produced by substituting tryptophan (W) for tyrosine (Y) at residue 113 and phenylalanine (F) for tryptophan (W) at residue 140. For each group, the proteins have the same amino acid at residue 140, but a different amino acid at residue 113. The solvent perturbation of nuclease fluorescence and 1,8-anilinoaphthalene-8-sulfonate binding studies showed that the substitutions do not change the side-chain positions of amino acids at residues 113 and 140. Chromatography of the proteins on the Phenyl-Superose HR5/5 column showed that the proteins with tryptophan at residue 113 have longer retention times than the proteins having tyrosine at residue 113; the proteins with the same amino acid at residue 113 have almost the same retention time regardless of substituting phenylalanine for tryptophan at residue 140. The studies clearly indicate that not all amino acid substitutions have an effect on protein retention; the contribution to retention of a given amino acid substitution depends on its position in a protein. Single amino acid substitutions at the exterior surface of a protein, which change the strength of hydrophobic interaction, can affect the protein retention in hydrophobic interaction chromatography. Staphylococcal nuclease and its mutants with only one amino acid difference on their surfaces can be discriminated by the phenyl-Superose column. The high resolving power of the Phenyl-Superose column makes it suitable not only for separating proteins, but also for providing a workable method for the analysis of the conformation homogeneity of refolded recombinant protein molecules by examining the hydrophobicity changes of protein molecules.

1. Introduction

Hydrophobic interaction chromatography (HIC) is a purification technique used to separate proteins on the basis of surface hydrophobicity [1,2]. Since HIC can preserve protein conformation and minimize denaturation, it is used to separate proteins that span a wide range of surface hydrophobicity. The role of protein structure in chromatographic behaviour has been studied by Regnier and co-workers [3,4]. They used different lysozymes isolated from related bird species as model proteins to determine the contribution of certain amino acid substitutions in the proteins to retention. They predicted that amino acid substitutions did not affect the size of

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the hydrophobic contact surface area, but rather the strength of hydrophobic interactions, and only those residues at or near the exterior surface of a protein would have a major impact on chromatographic behaviour. However, systematic studies of the relationships between protein structure and chromatographic behaviour in HIC are still in the early stages, and more direct evidence is needed. Staphylococcal nuclease (EC 3.1.4.7) is a small globular protein of 149 residues containing no disulfide bonds or cysteines. X-ray crystal analysis of the protein indicates that a single tryptophan at residue 140 is buried in the interior of the protein, a tyrosine at residue 113 is not involved in the formation of any intramolecular hydrogen bonds and its side-chain is located on the protein surface [5]. Here we can take advantage of the structural characteristics of the protein to create two groups of staphylococcal nucleases, Y113/W140 (wild-type), Y113W/W140 and Y113/W140F, Y113W/W140F, by substituting tryptophan (W) for tyrosine (Y) at residue 113, and phenylalanine (F) for tryptophan (W) at residue 140. For each group, the proteins have different amino acids at residue 113 and the same amino acids at residue 140. The proteins provide good materials for examining the effect of amino acid substitutions at different regions of a protein on chromatographic behaviour. In this paper, we focus on examining the effect of single amino acid residue substitutions on chromatographic retention and the extent to which hydrophobic differences on surface of proteins can be discriminated by HIC using staphylococcal nuclease and its mutants as model proteins. The possibility of using HIC to examine the conformation homogeneity of refolded recombinant protein molecules is also discussed.

2. Experimental

2.1. Materials

All chemicals were of analytical-reagent grade. 1-Anilinonaphthalene-8-sulfonate (1,8-ANS) was purchased from Sigma.

The phenyl-Superose HR5/5 column (1 ml) was obtained from Pharmacia Laboratory Separation Division, which is used with a Pharmacia fast protein liquid chromatographic (FPLC) system.

2.2. Generating mutants and purification of staphylococcal nucleases

Plasmid pBVS-2 for expression of the staphylococcal nuclease (Y113/W140) was constructed by Jing et al. [6]; the nuclease was overproduced as inclusion bodies in *Escherichia coli* cells under the transcriptional control of $P_R P_L$ promoters regulated by cI857 temperature-sensitive repressors. Three mutants of the nuclease with different amino acid substitutions at residue 113 or/and 140, named Y113W/W140, Y113/W140F and Y113W/W140F, were obtained by the site-directed mutagenesis method described by Kunkel et al. [7], and overproduced in *E. coli* cells under the same transcriptional control as described above. The nuclease and its mutants were purified from *E. coli* cells harbouring the appropriate recombinant plasmid. *E. coli* cells grown to an absorbance of around 0.5–0.6 at 600 nm at 30°C in LB broth were induced at 42°C for 3–5 h. Partial purification was performed according to Shortle's cold-ethanol precipitation method [8]. For further purification, the partially purified protein was resuspended in a buffer of 1 *M* Tris–HCl (pH 9.2)–2.5 m*M* EDTA at 4°C overnight, and chromatographed on a Bio-Rex 70 column (15 cm × 2.6 cm I.D.) once or twice according to Shortle's procedure [9]. The peak containing the protein was collected and dialysed against distilled water. The purified proteins appeared to be homogeneous as judged by sodium dodecylsulfate polyacrylamide gel electrophoresis.

2.3. Fluorescence measurements

Fluorescence measurements were made with a Hitachi F4010 spectrofluorimeter at 25°C. Solvent perturbation of nuclease fluorescence was carried out according to the method described by Cuatrecasas et al. [10]. The enhancement of

tryptophanyl fluorescence by ethanol is used as a structural probe. Each solution contained 8 μM nuclease in 20 mM Tris–HCl (pH 7.4)–0.1 M NaCl and 0–30% (v/v) of ethanol. The tryptophanyl fluorescence intensities were measured at 360 nm with an excitation wavelength of 280 nm. For 1,8-ANS binding studies, each sample contained 180 μM of 1,8-ANS and 16 μM of nuclease protein in 20 mM Tris–HCl (pH 7.4), the fluorescence spectrum was measured after incubation of the sample for 20 min at 25°C, the wavelength of excitation was 345 nm and the slit width was 10 nm.

2.4. *Circular dichroism*

CD spectra were obtained on a Jasco J-500A spectropolarimeter. Samples were scanned from 250 to 195 nm using a quartz cuvette with a pathlength of 1 mm at 20°C; the concentration of each sample was 0.4 mg/ml in 20 mM Tris–HCl (pH 7.4).

2.5. *Activity assays*

The enzyme activity was measured with a Shimadzu UV-250 spectrophotometer by monitoring the increase in absorbance at 260 nm on addition of enzyme to 1 ml of a solution containing 50 μg/ml of boiled salmon sperm DNA, 25 mM Tris–HCl (pH 7.4) and 10 mM $CaCl_2$ [11].

2.6. *Hydrophobic interaction chromatography on phenyl-Superose HR5/5*

Phenyl-Superose is a derivative of the rigid, cross-linked agarose-based gel Superose 12 and contains covalently bonded hydrophobic phenyl groups. The average particle size is 10 μm. Phenyl-Superose has a negligible amount of charged groups, ensuring true hydrophobic interaction chromatography. All the buffers and samples used in the chromatography were filtered through a 0.22-μm sterile filter. Each sample was dissolved in buffer A [50 mM potassium phosphate (pH 7.0)–1.7 M ammonium sulfate] to give a final concentration of 1 mg/ml. A 50-μg amount of each sample was injected into the column equilibrated with buffer A. Chromatography was performed with a linear gradient from 0 to 100% buffer B with buffer A [buffer B = 50 mM potassium phosphate (pH 7.0)] at room temperature. A gradient volume of 15 ml at a flow-rate of 0.5 ml/min was used for testing the retention time of each sample individually on the phenyl-Superose column. The effluent was monitored at 280 nm. When the sample was a mixture of Y113/W140 and Y113W/W140 or Y113/W140F and Y113W/W140F, a gradient volume of 20 ml at a flow-rate of 0.25 ml/min was used.

3. Results and discussion

3.1. *Positions of amino acids at residue 113 and 140 of the nucleases*

The X-ray crystal analysis of staphylococcal nuclease (Y113/W140) showed that a single tryptophan at residue 140 is buried in the interior of the protein and a tyrosine at residue 113 is located on its surface [5]. Fig. 1 shows the results of solvent perturbation of nuclease fluorescence; increasing the ethanol concentration from 0 to 30% produces a linear increase in the tryptophanyl fluorescence of Y113W/W140 and Y113W/W140F. In contrast, no effect is observed on the tryptophanyl fluorescence of Y113W/W140 (wild-type). As low-molecular-mass tryptophanyl compounds showed a significant increase in ethanol [12], it is clear that the tryptophan residues at residue 113 of the mutants are also located on the surface of proteins. Y113W/W140 shows a less significant tryptophanyl fluorescence change than Y113W/W140F due to ethanol perturbation, as it contains two tryptophanyl residues, which make the mutant have a higher background fluorescence at 360 nm without ethanol perturbation.

It is known that 1,8-ANS can serve as a probe for hydrophobic sites on proteins [13], so the fluorescence of 1,8-ANS bound to the nucleases will provide another evidence to support the results mentioned above. Fig. 2 shows the fluo-

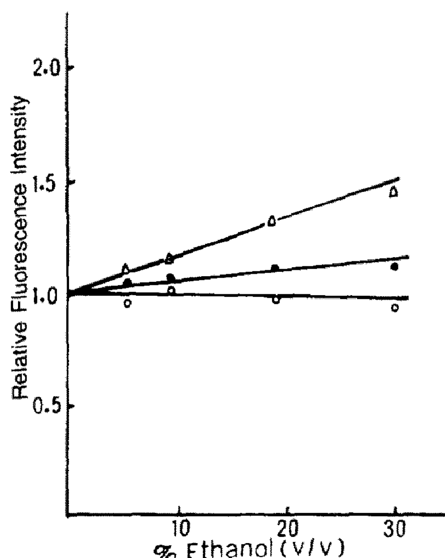

Fig. 1. Effect of ethanol on tryptophanyl fluorescence intensities of (○) staphylococcal nuclease and its mutants: (△) Y113W/W140F and (●) Y113W/W140. Solutions contained the nucleases (8 μM) in 20 mM Tris–HCl (pH 7.4)–0.1 M NaCl and the indicated amount of ethanol. The tryptophanyl fluorescence was measured at 360 nm with an excitation wavelength of 280 nm. For each protein, the fluorescence intensity in absence of ethanol was taken as 100%.

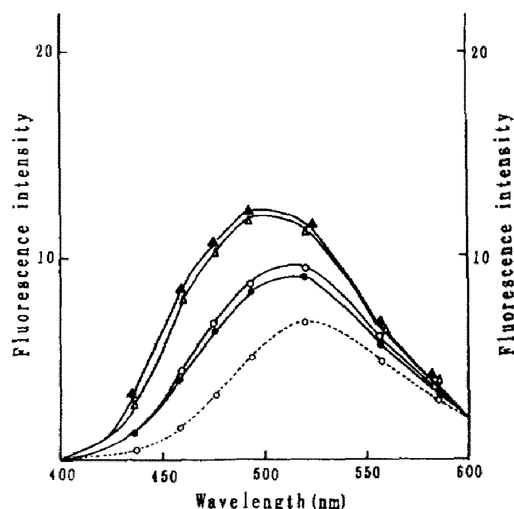

Fig. 2. Fluorescence emission spectra of 1,8-ANS in the presence of staphylococcal nuclease and its mutants. Spectra for 1,8-ANS with (●) Y113/W140, (○, solid line) Y113/Y140F, (△) Y113W/W140 and (▲) Y113W/W140F and (○, dashed line) a control with no protein. Each sample contained 180 μM of 1,8-ANS and 16 μM of the nuclease in 20 mM Tris–HCl (pH 7.4). The fluorescence spectra were measured after incubation of the samples for 20 min at 25°C; the wavelength of excitation was 345 nm.

rescence spectra of 1,8-ANS bound to the nuclease and its mutants. The fluorescence intensities of 1,8-ANS bound to the nucleases having same amino acid at residue 113 are almost the same regardless of the substitution of phenylalanine for tryptophan at residue 140. When 1,8-ANS is bound to the nucleases having tryptophan at residue 113, there is an apparent increase in fluorescence intensity and a blue shift of the fluorescence maximum compared with the fluorescence of 1,8-ANS bound to the nucleases with tyrosine at residue 113. The results again indicate that the tryptophan at residue 113 is located on the surface of proteins. The increase in fluorescence intensity and the blue shift of the fluorescence maximum occur because tryptophan has a higher hydrophobicity than tyrosine [14]. In addition, the results also show that the substitution of phenylalanine for tryptophan at residue 140 does not affect the hydrophobic properties of the proteins, which means that the phenylalanine at residue 140 is buried in the interior of the proteins.

3.2. Effect of single amino acid substitution on protein retention in HIC and resolution of phenyl-Superose HR5/5 column

As described above, the effect of amino acid substitutions on the chromatographic retention of proteins was analysed by utilizing staphylococcal nuclease and its mutants. As shown in Fig. 3, the proteins with tryptophan at residue 113 were eluted at longer retention times than those having tyrosine at residue 113; the proteins with the same amino acid at residue 113 have almost the same retention time regardless of substituting phenylalanine for tryptophan at residue 140. It is unlikely that the differences in retention time between the nucleases are mainly caused by gross conformational changes as all the nucleases have almost the same CD spectra (Fig. 4) and

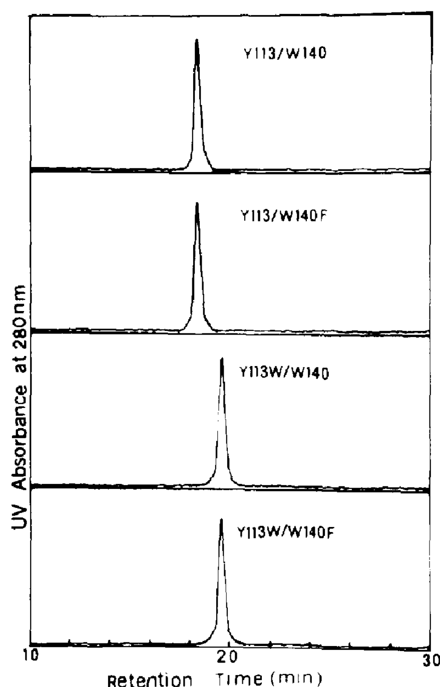

Fig. 3. Chromatographic retentions of staphylococcal nuclease (Y113/W140) and its mutants (Y113W/W140, Y113/W140F and Y113W/W140F) on the phenyl-Superose HR5/5 column. Chromatography was performed with a linear gradient (15 ml) from 0 to 100% buffer B with buffer A at room temperature. The effluent was monitored at 280 nm. Mobile phase: buffer A, 50 mM potassium phosphate (pH 7.0)–1.7 M ammonium sulfate; buffer B, 50 mM potassium phosphate (pH 7.0). Flow-rate, 0.5 ml/min.

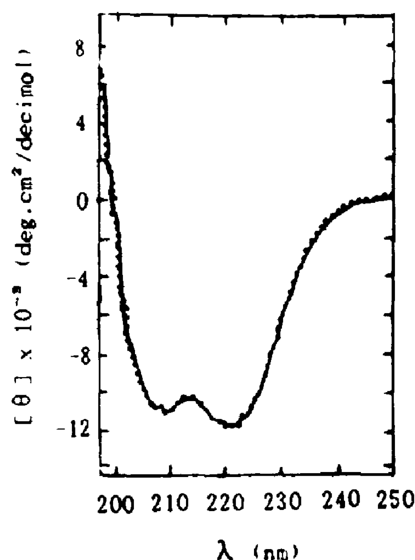

Fig. 4. Circular dichroism spectra of staphylococcal nuclease: Y113/W140 (solid line) and its mutants Y113W/W140, Y113W/W140F and Y113/W140F (dotted line). Refer to the Experimental section for the conditions of the spectra.

the same activities (about 1000 units/mg), so the reason is that the substitution of tryptophan for tyrosine at residue 113 makes the nucleases with Y113W more hydrophobic than the nucleases with Y113 on their surfaces. Hence they would undergo stronger hydrophobic interactions with the Phenyl-Superose adsorbent. Of course, subtle changes in local conformation between the proteins caused by the substitution of tryptophan for tyrosine at residue 113 probably make some contribution to retention. However, there is no change in retention time when tryptophan at residue 140 is changed to phenylalanine, although phenylalanine is less hydrophobic than tryptophan [14]. A reasonable explanation is that the substitution does not make any changes in the hydrophobicity on their surfaces as the phenylalanine is buried in the interior of the mutants as described above. The studies clearly indicate that the contribution to retention of a given amino acid substitution depends on its position in the protein. Single amino acid substitution at the exterior surface of a protein can affect its retention in HIC if it changes the strength of the hydrophobic interactions.

In order to examine further what differences in hydrophobicity at the protein surface can be discriminated by HIC on the phenyl-Superose column, a mixture of Y113/W140 and Y113W/W140 or Y113/W140F and Y113W/W140F was applied to the phenyl-Superose column using the conditions described under Experimental. The proteins with only one amino acid difference on their surfaces can be well separated (Fig. 5). In agreement with earlier studies [4], the variant separation mechanism involves specific protein surface contact with the adsorbent in the region of primary structure protein differences. This indicates that the phenyl-Superose column has a high resolving power, and the properties of phenyl-Superose HIC can be used not only to

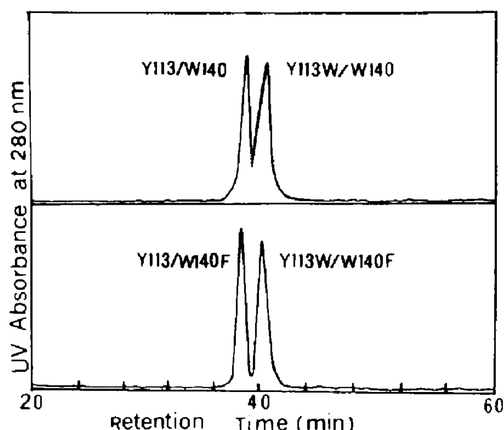

Fig. 5. Chromatography of the mixtures of Y113/W140 (wild-type) and Y113W/W140 or Y113/W140F and Y113W/W140F on the phenyl-Superose HR5/5 column. Chromatographic conditions in Fig. 3 except that the gradient volume was 20 ml at a flow-rate of 0.25 ml/min.

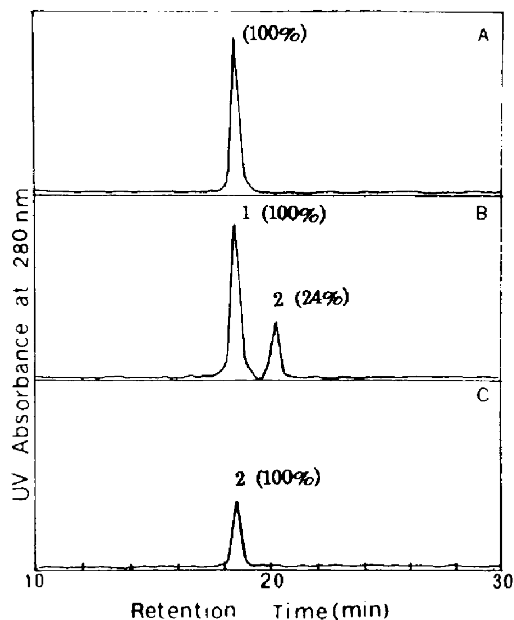

Fig. 6. Examination of the conformation homogeneity of the recombinant-derived staphylococcal nuclease by using HIC on the phenyl-Superose HR5/5 column. Chromatography was performed under the conditions described in Fig. 3. (A) Native nuclease; (B) nuclease purified from inclusion bodies; sometimes two peaks were eluted from the column; (C) re-chromatography of peak 2 on the column after denaturation and renaturation treatment. The values in parentheses represent the relative activities of the nucleases in different peaks.

separate proteins but also to determine the relative hydrophobic characters of closely related proteins.

As numerous proteins have been shown to accumulate in an insoluble form when they are highly expressed in *E. coli* cells by the recombinant DNA technique, the protein of interest may be solubilized and require refolding to restore to it the same biological activity as for native protein after renaturation. However, there is no guarantee that the conformation among these soluble protein molecules is homogeneous. Therefore, finding a convenient way to discriminate the correctly folded molecules from the soluble but improperly folded molecules will be significant in both basic and applied research. The following experimental results may show the possibility of examining the conformation state of refolded recombinant proteins by using the phenyl-Superose column.

When the nuclease, which was purified from inclusion bodies as described above, was analysed on the HIC column, two peaks were sometimes eluted (Fig. 6B). Peak 1 has the same retention time and enzyme activity as the native enzyme, whereas peak 2 has a longer retention time and only 24% activity of the native enzyme. However, only one peak with the same retention time and activity as the native enzyme was obtained after peak 2 was further denatured in 4 *M* urea and renatured by dialysis against distilled water (Fig. 6C). It seems that peak 1 represents the correctly folded molecules of the enzyme and peak 2 represents the soluble but improperly folded molecules of the enzyme. As HIC is used to separate proteins on the basis of the surface hydrophobicity of proteins [1,2], the differences in retention time between molecules of the nuclease indicate that there are some differences in hydrophobicity between peaks 1 and 2, which ought to be caused by a conformational or structural change (e.g., improper folding) during the denaturation–renaturation process. Although refolding is easily achieved with some small polypeptides, there are no general rules for efficient refolding, and consequently appropriate conditions must be determined by trial and

error. Sometimes for recombinant proteins, especially for some proteins with high molecular masses and disulfide bonds, even when denaturation–renaturation treatment is carried out with great care there is still a small proportion of soluble molecules with improper folding. As long as there is a certain difference in surface hydrophobicity among them, in specific cases HIC may provide a workable method for the analysis of conformation homogeneity of recombinant proteins.

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