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Protein sorting by high-performance liquid chromatography

I. Biomimetic interaction chromatography of recombinant human deoxyribonuclease I on polyionic stationary phases*

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ABSTRACT

Chromatographic separations can be tailored to exploit specific interactions between a stationary phase ligand and a protein structural feature of interest. Variations in this feature then form the basis for sorting a mixture of closely related proteins into defined subpopulations. This report describes the sorting of variants of recombinant human deoxyribonuclease I (rhDNase) that differ in the occurrence of deamidation at a single residue. rhDNase, an enzyme that non-specifically hydrolyzes DNA, is glycosylated and exhibits considerable charge heterogeneity owing to the sialylation and phosphorylation of its N-linked oligosaccharides. This heterogeneity obscures the relatively subtle differences between deamidated and intact rhDNase, preventing separation on this basis in conventional ion-exchange HPLC. Published structural information on bovine DNase

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reveals that the analogous labile asparagine residue is involved in DNA binding, so stationary phases containing polyanionic ligands mimicking nucleic acids were employed to separate the deamidation variants of rhDNase. Electrostatically immobilized DNA, a "tentacle" cation exchanger (TCX) and immobiliid heparin columns all resolved the deamidated and intact forms of rhDNase when operated at pH 4.5. The ligands of the TCX and heparin columns are sufficiently long, flexible and polyanionic to interact with rhDNase in a manner similar to DNA and to sort rhDNase variants on the basis of the charge difference of a single residue involved in that interaction. A non-hydrolyzable double-stranded oligonucleotide analogue of DNA was also synthesized and immobilized to an HPLC support. This column, operated at pH 6, where rhDNase is active, resolved the two isomeric products of deamidation of rhDNase, i.e., variants of the enzyme containing either aspartate or isoaspartate in lieu of asparagine at the deamidation site in rhDNase. This is the first reported separation of intact variants of a glycoprotein differing on the basis of these isomeric products of deamidation through the common cyclic imide mechanism.

INTRODUCI'ION

Proteins are large, complex molecules containing a myriad of combinations of chemically distinct amino acid side chains. In addition, certain proteins bear post-translational modifications that further compound the intricacy of the molecule and that may, in the case of glycosylation, convert the protein into a variegated amalgam of species differing in the structures of the conjugate carbohydrates. The analytical characterization of a protein is, therefore, hampered by the multiplicity of chemical species that contribute to its physico-chemical behavior. The most prominent example of this approach to protein separations is affinity chromatography [1] that exploits a biological interaction between the protein and its natural ligand. The specificity of these interactions affords a powerful means of separating a protein from non-binding species.

Affinity chromatography is, however, just one example of a general approach to high resolution separations of proteins in which the chromatographic conditions are chosen to be selective for a specific feature of a protein, to give high selectivity for that feature, and to exhibit little or no selectivity for other characteristics. Hence, as in affinity separations, the chromatographic conditions are adjusted so that the stationary phase selectively interacts with a single or few chemical moieties of the protein, ignoring the remaining large number of distinct chemical species. The value of this approach to chromatographic separations is that a nonselective analytical system would be swamped by the variety of species present on a protein and yield little or no selectivity for any particular characteristic. The general approach to separating protein species on the basis of a specific single characteristic is termed "protein sorting", by analogy to the post-translational trafficking of proteins on the basis of specific structural motifs or "signals" on the protein that target it to particular intracellular or extracellular locations [2]. The power of chromatographic protein sorting is apparent when populations of closely related species are sorted, for instance according to the presence of particular carbohydrate groups on N-linked glycans [3] or, in the example described in this report, according to the occurrence of deamidation at a single site in a glycoprotein, recombinant human deoxyribonuclease I (rhDNase), that exhibits considerable charge heterogeneity engendered by glycosylation.

Deoxyribonuclease I (DNase) is an endonuclease that non-specifically hydrolyzes DNA. DNase isolated from bovine pancreatic tissue has been extensively characterized [4–6] and its crystal structure has been solved [7]. Human DNase has been cloned and expressed in mammalian cells for use as a therapeutic in the treatment of cystic fibrosis [8]. rhDNase has a high degree of charge heterogeneity arising from phosphorylation and sialylation of carbohydrate structures linked to both Asn-18 and Asn-106. The charge heterogeneity is manifested, for example, in isoelectric focusing (IEF) where rhDNase forms a ladder of six or more bands in a *pI* range of 3-4, as shown in Fig. 1. Additional charge complexity accrues to rhDNase through deamidation of the Asn-74 residue in the molecule. The deamidation of asparagine residues is well characterized in proteins [9]. Asparagine–serine sequences, such as that of residues 74 and 75 in rhDNase, are particularly prone to deamidate under alkaline conditions through a cyclic

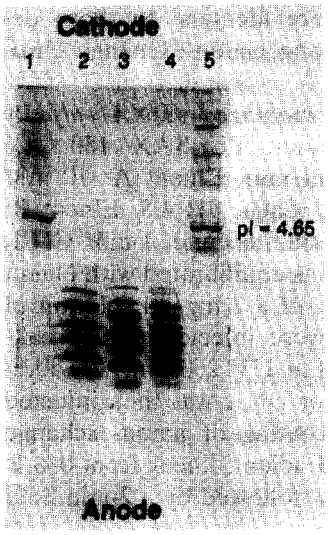

Fig. 1. Isoelectric focusing gel showing (lane 2) rhDNase, (lane 3) its deamidated variant and (lane 4) an equimolar admixture of the two components. Lanes 1 and 5 contained p*I* markers. The 0.2 mm thick IEF gel contained a 2% ampholyte mixture (Serva) in a pH range of 3-5 and was stained with Coomassie blue. Focusing was performed for 2 h with a voltage limit of 1600 V, and a power limit of 10 W.

imide intermediate [10] to yield two products, Asp-74 and iso-Asp-74. Fig. 2 shows the reaction pathway followed in this conversion of a neutral side chain to two isomeric residues with acidic side chains. The two isomeric products of deamidation differ in the lengths of both the side chain and the polypeptide backbone, with the iso-Asp form containing an added methylene group in the backbone. rhDNase has been shown to undergo deamidation at Asn-74 under conditions of elevated pH and temperature, and to evince the reaction in tryptic mapping analysis [11]. IEF analysis of the deamidated variant of rhDNase, shown in Fig. 1, demonstrates that it retains the carbohydrate-related charge heterogeneity of the non-deamidated glycoprotein. IEF analysis of an admixture of the two variants, shown in lane 3 of Fig. 1, results in an IEF pattern with 12 or more bands, revealing the subtle acidic shift of the bands of the deamidated protein. The small difference between rhDNase and its deamidated variant -in terms of both the structural difference that is limited to conversion of an amide side chain to a carboxylic side chain as well as the small acidic shift noted in IEF- in the midst of a relatively large degree of charge heterogeneity of the glycoprotein, portends difficulties in resolving the variant from intact rhDNase by chromatographic methods. This report describes studies of the behavior of rhDNase and its deamidated variant in a variety of ion-exchange HPLC systems, including those designed to mimic the interaction of rhDNase with DNA, its natural substrate. The requirements for sorting of rhDNase variants on the basis of the occurrence of deamidation at Asn-74, and the protein structural basis for these requirements, are described.

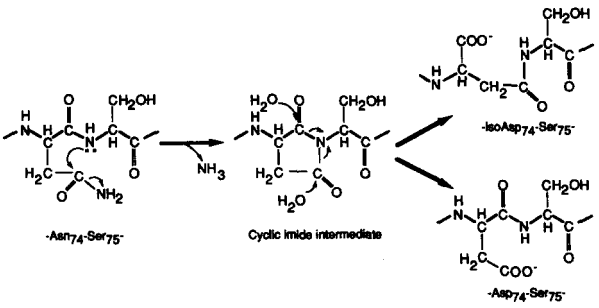

Fig. 2. Schematic of the base-catalyzed deamidation pathway for the Asn-Ser sequence in rhDNase. Asparagine residues deamidate under alkaline conditions by a mechanism that proceeds through a cyclic imide intermediate. The intermediate hydrolyzes to yield one of two isomeric products, containing either aspartic acid or isoaspartic acid residues in lieu of asparagine.

EXPERIMENTAL

Chromatographic equipment

All chromatography was performed on a Hewlett-Packard (Palo Alto, CA, USA) Model 1090M liquid chromatograph equipped with a diode array detector, autosampler and Chemstation data collection and analysis software.

Materials

rhDNase and its deamidated variant were produced by mammalian cell culture and purified at Genentech. N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES), potassium phosphate and salmon testes DNA were obtained from Sigma (St. Louis, MO, USA) and sodium acetate and sodium chloride from Mallinckrodt (Paris, KY, USA). (2-N-Mor-

pholino)ethanesulfonic acid (MES) was obtained from ICN Biochemicals (Costa Mesa, CA, USA). Empty stainless-steel columns with dimensions of 50 mm x 4.6 mm I.D. were purchased from Alltech (Deertield, IL, USA). Hydrosphere EP epoxide silica resin was obtained from Rainin (Wobum, MA, USA). The 5-μm LiChrosphere SO_3^- packing with pore diameters of 1000 Å was obtained packed in glass cartridges that were unpacked, and the resin defined by suspending in 5 m*M* sodium acetate, pH 4.5. The tines remaining in the suspension after settling for 20 min were poured off. This procedure was repeated five times prior to packing the material into a steel column in the same buffer. The PL SAX, 150 x 4.6 mm I.D., 8 μm, 1000 Å strong anion-exchange column was purchased from Polymer Labs. (Foster City, CA, USA). The TSK SP 5PW and TSK Heparin 5PW columns both having dimensions of 75 mm × 7.5 mm I.D. were purchased from HP Genenchem (Palo Alto, CA, USA).

Methods

Strong anion-exchange chromatography. Column: Polymer Labs SAX, 150 x 4.6 mm. Flow-rate: 1.0 ml/min. Eluent A: 5 m*M* HEPES, 1 m*M* $CaCl_2$ pH 7.0. Eluent B: 1 *M* NaCl, 5 m*M* HEPES, 1 m*M* $CaCl_2$ pH 7.0. Gradient: 1-min hold at 0% B, followed by a linear gradient to 50% B over 40 min.

Strong ***cation-exchange chromatography.*** Column: TSK SP 5PW 75 x 7.5 mm. Flow-rate: 1.0 ml/min. Eluent A: 10 m*M* sodium acetate, 1 m*M* $CaCl_2$ pH 4.5. Eluent B: 1 M NaCl, 10 m*M* sodium acetate, 1 m*M* $CaCl_2$ pH 4.5. Gradient: 4-min hold at 0% B, followed by a linear gradient to 58% B over 26 min.

"Tentacle" cation-exchange chromatography. Column: LiChrosphere SO;, 5 μm, 1000 Å packed into a 50 x 4.6 mm I.D. stainless-steel column as described above. The chromatographic conditions were identical to those described above for strong cation-exchange chromatography.

Heparin column chromatography. Column: TSK Heparin 5PW 75 x 7.5 mm. The chromatographic conditions were identical to those described above for strong cation-exchange chromatography.

Electrostatically immobilized DNA affinity chromatography. Column: PL SAX 150 × 4.6 mm. Flow-rate: 1.0 ml/min. Eluent A: 10 m*M* sodium acetate, 1 m*M* $CaCl_2$ pH 4.5. Eluent B: 1 *M* NaCl, 10 m*M* sodium acetate, 1 m*M* $CaCl_2$ pH 4.5. The column was equilibrated with eluent A and 250-μl aliquots of a 2 mg/ml solution of salmon testes DNA were injected until breakthrough was observed at UV 260 nm. An additional 10-mg amount of DNA was then injected to ensure adequate coating of anion-exchange sites in the column. Gradient: linear from 0% B to 70% B over 10 min, followed by a 2-min hold at 70% B.

Synthesis of non-hydrolyzable DNA analogue. Complementary phosphorothioate oligonucleotides with a sequence of 5′-GGCGCCT-CCAGCGTCGACGGCGNH$_2$-3′ and 5′-CG-CCGTCGACGCTGGAGGCGCC-3' were synthesized on aminated controlled-pore glass (Clontech, Palo Alto, CA, USA) using H-phosphonate chemistry [12] on a Model 8600 DNA synthesizer (Milligen/Biosearch, South San Francisco, CA, USA). The non-hydrolyzable DNA analogue was synthesized with an amino group at the 3' end of one of the oligonucleotide strands to allow coupling to an activated packing material. The two strands were annealed by diluting the mixture to 0.2 mg/ml with 10 m*M* Tris–HCl, 1 m*M* EDTA, pH 7.5. The mixture was then boiled for 1 min and slowly cooled to room temperature. Three volumes of absolute ethanol and 100 m*M* NaCl were then added to precipitate the double-stranded oligonucleotide. The oligonucleotide was then centrifuged and lyophilized.

Immobilization of oligonucleotide analogue. A 10-mg amount of double-stranded oligonucleotide was mixed with 1 g of epoxy silica resin and resuspended to 5 ml with 100 m*M* KH_2PO_4, pH 7.0. The mixture was shaken for 24 h at room temperature and then slurry packed into a 50 × 4.6 mm stainless-steel HPLC column.

DNA-analogue affinity chromatography. Eluent A: 5 m*M* MES, 1 m*M* $CaCl_2$, pH 6.0.

Eluent B: 1 M NaCl, 5 m*M* MES, 1 m*M* $CaCl_2$ pH 6.0. Gradient: linear from 0 to 100% B over 20 min. Flow-rate: 1.0 ml/min.

RESULTS AND DISCUSSION

Deamidation is a relatively common degradation reaction [9] that increases the number of charged residues on a protein at physiological pH and therefore, in many cases, is readily detected. The charge difference between a deamidated protein and its intact parent provides the basis for separation and analysis of the occurrence of deamidation by anion-exchange HPLC [13], cation-exchange HPLC [14], capillary electrophoresis [13] and isoelectric focusing electrophoresis [13]. In addition, tryptic mapping [15], mass spectrometry [15], N-terminal sequencing [16] and enzymatic methyl-accepting capacity [17] can provide information on the occurrence of deamidation in peptides and proteins,

In many cases, chromatographic methods that separate deamidation variants of intact proteins are preferable, since they are relatively fast and quantitative, can be automated and can be scaled up for preparative applications. The isoelectric point (p*I*) of the protein of interest often dictates the pH range of the mobile phase employed in ion-exchange HPLC and the choice of an anion or cation exchanger as the stationary phase. rhDNase is an acidic protein with a p*I* ranging between 3 and 4. Hence, anion-exchange chromatography is expected to provide strong retention under conditions near neutral pH where rhDNase is most stable. This mode of HPLC has been employed to resolve the deamidated variants of other acidic proteins [13]. A comparison of rhDNase and is deamidated variant run on anion-exchange chromatography is shown in Fig. 3A and B, respectively. Injection of an admixture of these two forms, shown in Fig. 3C, reveals no separation of the variants. Two fractions were collected from the admixture injection and run on an isoelectric focusing gel. The bands observed on the gel demonstrate that the column exhibits no selectivity for the deamidated form of rhDNase. Instead, the more acidic isozymes are retained longer on the anion exchanger, without resolving deamidation variants. The broad peaks obtained in the chromatograms shown in Fig. 3 probably reflect the charge heterogeneity con-

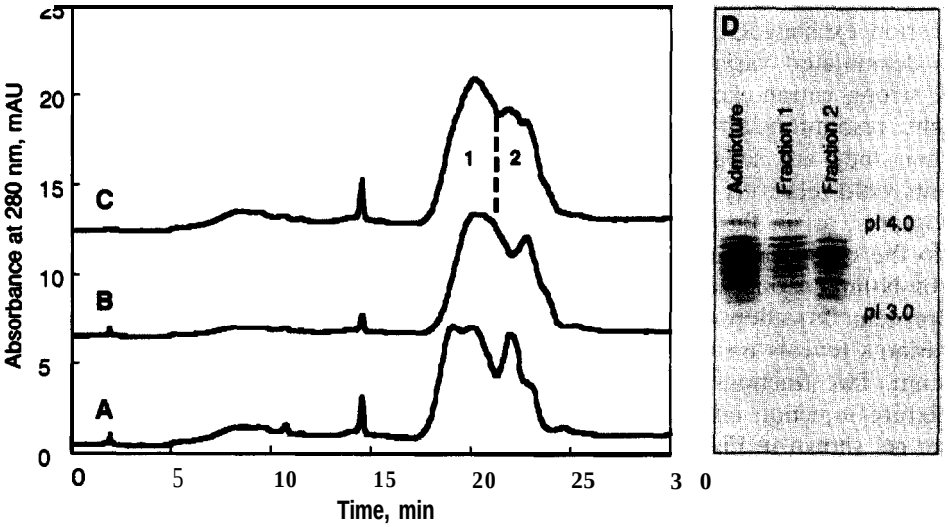

Fig. 3. Strong anion-exchange chromatography of (A) rhDNase, (B) its deamidated variant and (C) an admixture of the two variants. Fractions 1 and 2 were collected and run on a pH 3-5 isoelectric focusing gel shown in (D). Conditions are given in the text.

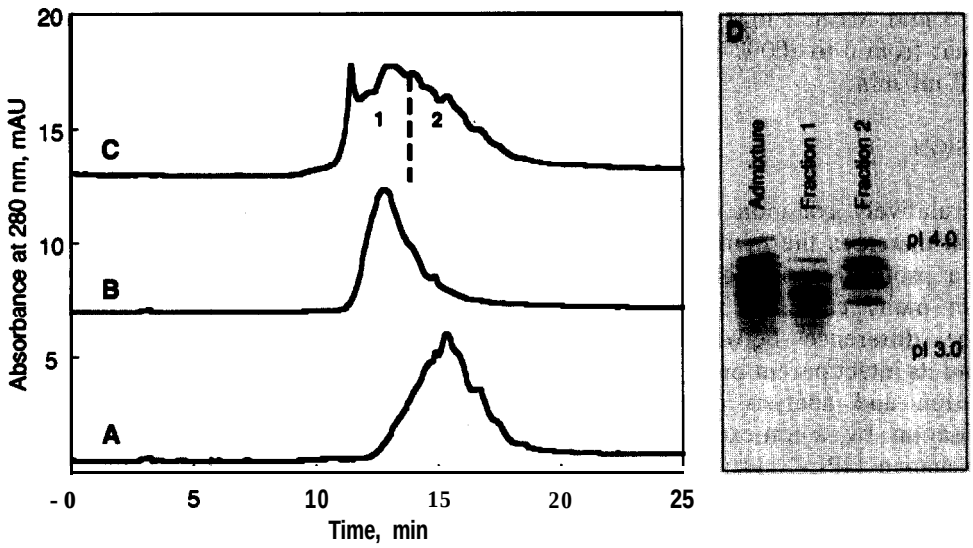

Fig. 4. Strong cation-exchange chromatography on a sulfopropyl column of (A) rhDNase, (B) its deamidated variant and (C) an admixture of the two variants. Fractions 1 and 2 were collected and run on a pH 3-5 isoelectric focusing gel shown in (D). Conditions are given in the text.

ferred on the protein by the presence of sialic acid and mannose phosphate on the glycans linked to rhDNase.

Cation-exchange HPLC has been employed to resolve the deamidated form of a glycoprotein that similarly exhibited a high degree of carbohydrate charge heterogeneity [14]. At pH 4.5, rhDNase, despite its low p*I*, can be retained on and recovered from a cation exchanger. Fig. 4 shows rhDNase and its deamidated variant injected on a sulfopropyl strong cation-exchange HPLC column. A slight resolution of these variants is observed when they are separately injected. rhDNase variants yield relatively broad peaks in these chromatograms, as on the anion exchange column, due to the charge heterogeneity of the population of N-linked oliosaccharides found on the glycoprotein. Injection of the admixture of the two variants reveals no useful separation with this column. Two fractions were collected from the admixture injection and run on an isoelectric focusing gel shown in Fig. 4D. As in anion-exchange chromatography, the separation is primarily based on carbohydrate charge heterogeneity with the more acidic species eluting earlier. The column exhibits a small selectivity for the deamidated rhDNase variant, but this selectivity is obscured by the contributions of sialylation and phosphorylation of the molecule to retention.

The residue in rhDNase that is prone to deamidation has been identified as asparagine-74 (Asn-74). According to the published crystal structure [7], the corresponding residue in bovine DNase is known to be involved in binding of the DNA substrate. Fig. 5 shows the bovine DNase residues that are involved in binding DNA as determined by cocrystallization with a

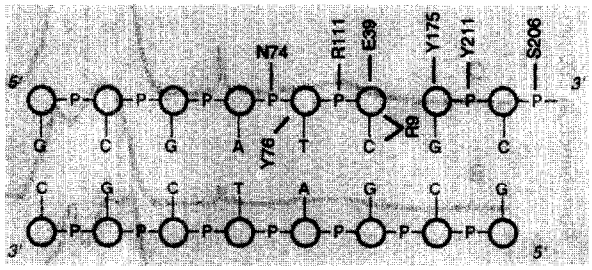

Fig. 5. Schematic of the DNA–DNase contacts determined from the crystal structure of the complex between a synthetic oligonucleotide and bovine DNase [7]. Asparagine-74 (N74) forms a hydrogen bond with a phosphate group in DNA. The other residues in the bovine enzyme involved in DNA-binding near the primary hydrolysis site on the substrate are arginines-9 and 111 (R9, R111), glutamate-39 (E-39), tyrosines-76, -175 and -211 (Y76, Y175, Y211) and serine-206 (S206).

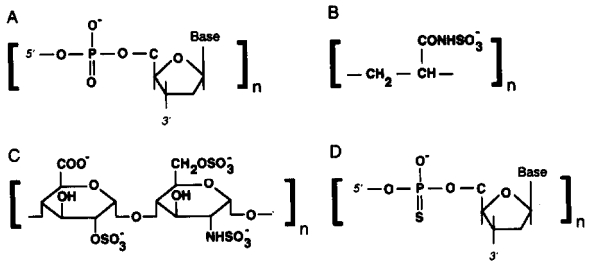

Fig. 6. Repeating units of the polyanionic ligands used for separation of rhDNase from its deamidated variant. (A) DNA, (B) tentacle SO_3^- (TCX), (C) heparin, (D) synthetic non-hydrolyzable DNA analogue.

synthetic oligonucleotide [7]. The Am-74 residue, that is conserved between human and bovine DNases, is shown to hydrogen bond with a phosphodiester group in the oligonucleotide backbone. Since deamidation occurs in a portion of the molecule that is accessible to DNA, the separation of rhDNase from its deamidated variant may be achieved by exploiting the biomimetic interaction of a polyanionic ligand that similarly can gain access to the relevant portion of the molecule. Fig. 6A shows the repeating unit of the DNA biopolymer. The phosphodiester linkages between repeating units confer multiple negative charges to the DNA polymer backbone. Fig. 6B–D shows the structures of polyionic ligands that were examined for -the potential to imitate DNA by accessing the DNA binding site of rhDNase, and separate deamidation variants of the molecule.

The repeating unit of the "tentacle" strong cation-exchange (TCX) resin is shown in Fig. 6B. The TCX ligand contains sulfate groups as does the conventional sulfopropyl column discussed above. The TCX ligand, however, contains multiple cation-exchange moieties strung along the polymeric backbone creating a flexible polyanionic ligand. Each ligand is reported to consist of 5–50 repeating units [18]. The polymeric nature of the cation exchanger imparts certain characteristics of DNA to the ligand. Fig. 7A and B show the chromatograms obtained for rhDNase and its deamidated variant, respectively, on the TCX column. Injection of the admixture of the two variants, shown in Fig. 7C, results in baseline resolution of the two species. The two peaks were collected from the chromatogram shown in Fig. 7C and run on an isoelectric focusing gel, shown in Fig. 7D. The IEF analysis confirms that the TCX column recognizes the single charge difference at residue 74 between the two variants of rhDNase. Hence the TCX column biomimetically interacts with the

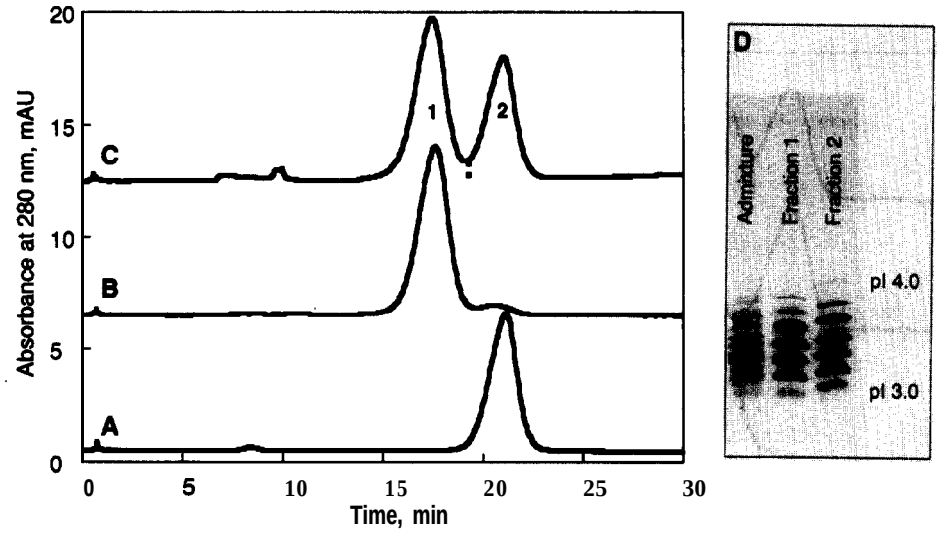

Fig. 7. Tentacle cation-exchange chromatography of (A) rhDNase, (B) its deamidated variant and (C) an admixture of the two variants. Fractions 1 and 2 were collected and run on a pH 3-5 isoelectric focusing gel shown in (D). Conditions were identical to those employed in Fig. 4.

same portion of the enzyme as the DNA substrate.

Another commercially available ligand containing a polymeric cation-exchange ligand, shown schematically in Fig. 6C, is the immobilized heparin column. Heparin is a sulfated glycosaminoglycan polysaccharide. Fig. 8A and B shows that the heparin column resolves rhDNase and its deamidated variant in a similar fashion to the TCX column. The efficiency of this separation is slightly less than that observed on the TCX column, although the interaction is still specific for the portion of the molecule containing the charge difference at residue 74. The two peaks resulting from injection of the admixture of the two variants (Fig. 8C) were collected and run on an isoelectric focusing gel shown in Fig. 8D. The resulting pattern demonstrates the selectivity exhibited by biomimetic interaction chromatography with this column despite the considerable carbohydrate-engendered charge heterogeneity of the two species.

The analyses of rhDNase variants on the TCX and heparin columns were carried out at pH 4.5 in order to obtain sufficient retention of the acidic glycoprotein. In order to demonstrate that the TCX and heparin ligands mimic DNA in the separations shown in Figs. 7 and 8, a DNA affinity column was prepared by electrostatic immobilization of salmon testes DNA on an anion-exchange HPLC column. The hydrolysis of DNA by DNase is negligible at pH 4.5, so under these conditions a practical DNA affinity column can be prepared. The strong anion-exchange column was coated with DNA at pH 4.5, effectively converting it to an affinity column for rhDNase. The admixture of rhDNase with its deamidated variant was injected, yielding the two peaks shown in the chromatogram in Fig. 9. The two peaks were shown to be the two charge

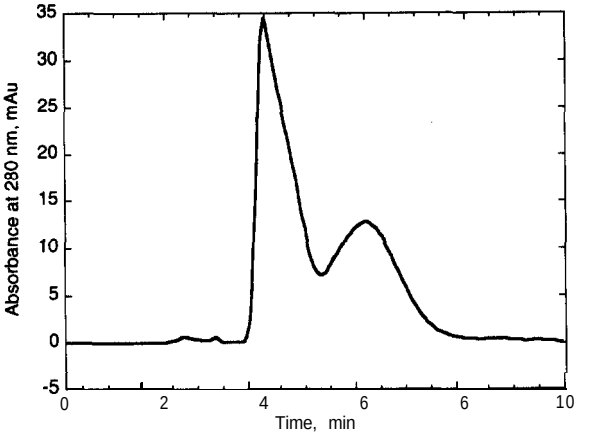

Fig. 9. Electrostatically immobilized DNA affinity chromatography of an admixture of rhDNase and its deamidated variant. Conditions are given in the text.

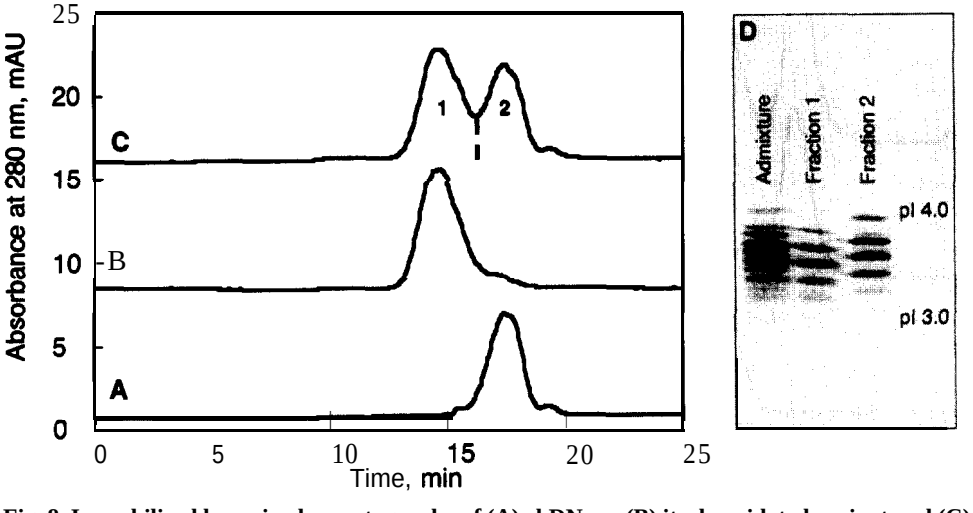

Fig. 8. Immobilized heparin chromatography of (A) rhDNase, (B) its deamidated variant and (C) an admixture of the two variants. Fractions 1 and 2 were collected and run on a pH 3-5 isoelectric focusing gel shown in (D). Conditions were identical to those employed in Fig. 4.

variants of rhDNase by collection and reinjection on the TCX column (data not shown), with the deamidated form eluting from the column earlier than intact rhDNase. This separation could not be obtained on the bare anion-exchange column prior to coating with DNA. The DNA affinity column exhibited relatively poor efficiency and reproducibility, owing to the poor stability of the electrostatic immobilization and the heterogeneity of the salmon testes DNA preparation. Nevertheless, the separation obtained on this column confirms the involvement of the labile Asn-74 residue in binding DNA, and the biomimetic behavior of the TCX and heparin ligands under these conditions.

Another way to investigate the interaction between DNA and rhDNase variants is to employ a column containing an immobilized synthetic oligonucleotide. In this study, complementary phosphorothioate analogues [12], having the linkage shown in Fig. 6D, were synthesized with the sequences given in the Experimental section. One strand was synthesized with a free amino group at the 3' end, and the annealed strands were covalently attached to activated silica particles through this group to make the immobilized double-stranded affinity column. At pH 4.5 the separation by this column was similar to that of the electrostatically immobilized DNA column (data not shown). The phosphorothioate DNA analog is refractory to hydrolysis by DNase [19], and so can be operated with a higher-pH mobile phase under conditions where the DNA affinity column is hydrolyzed. Fig. 10 shows the chromatograms of rhDNase, the deamidated form of rhDNase and the admixture of the two species on the oligonucleotide affinity column operated at pH 6. The column resolves the deamidated and intact forms of rhDNase, but also separates the deamidated form of the enzyme into two peaks. The two peaks were collected and characterized by tryptic mapping. Fig. 11 shows the tryptic maps of the two peaks of deamidated rhDNase collected from the oligonucleotide column, along with the map of intact rhDNase. The tryptic maps differ in the T6-7 and T7 peptides that contain the labile Asn-74 residue. The T7 peptide contains Asn as residue 74 and hence is an indicator of intact rhDNase, while

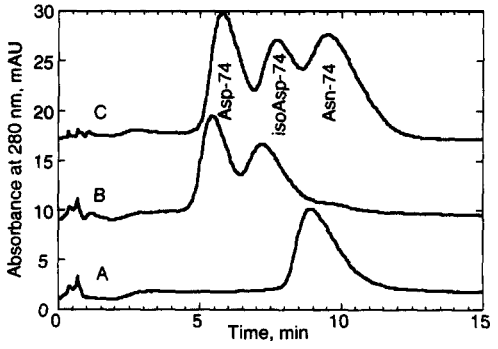

Fig. 10. Non-hydrolyzable DNA analogue affinity chromatography of (A) rhDNase, (B) its deamidated variant and (C) an admixture of the two variants. The peaks labeled "Asp-74", "isoAsp-74" and "Asn-74" were collected for characterization by tryptic mapping. The first peak was found to be rhDNase with aspartate at residue 74 (Asp-74 rhDNase), the second peak to be the isoAsp-74 isomer of the deamidated variant and the third peak to be intact rhDNase containing asparagine at position 74.

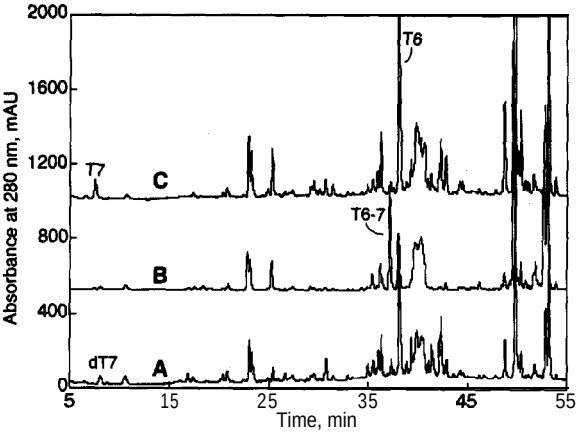

Fig. 11. Tryptic maps of the fractions collected from the DNA analogue affinity separation shown in Fig. 10C. (A) Chromatogram of the peptide mixture obtained on tryptic digestion of peak Asp-74, the deamidation variant containing aspartic acid at residue 74, as indicated by the peptide labeled dT7. (B) Chromatogram of the digest of peak isoAsp-74, the deamidation variant containing isoaspartic acid at residue 74, as indicated by the peptide T6-7. (C) Chromatogram of the digest of peak Asn-74, the intact form of rhDNase that contains asparagine at residue 74. The T7 peptide includes residues 74-77 of the digested protein, with asparagine at residue 74. The dT7 peptide similarly includes residues 74-77, but with aspartic acid at residue 74. The T6-7 peptide includes residues 51-77, with isoaspartic acid at residue 74 and no cleavage of the peptide bond after the arginine at residue 73.

the dT7 and T6-7 peptides are markers for the Asp-74 and isoAsp-74 containing deamidation variants, respectively. The tryptic maps thus reveal that the oligonucleotide analog column resolves the isomeric forms of deamidated rhDNase that contain either an aspartic acid or an isoaspartic acid residue in lieu of asparagine at position 74. The structural differences among these variants are shown in Fig. 2. The side chains of the two products of asparagine deamidation have essentially identical p*K* values, and so do not differ in charge, and rhDNase variants differing in these isomeric side chains are not resolved by isoelectric focusing gel electrophoresis. Nevertheless the two forms of deamidated rhDNase, that differ in both the length of the side chain and in the length of the polypeptide backbone, display sufficient differences in binding to the high-performance affinity column to allow their separation. This is apparently the first example of the separation of the two isomers of an intact glycoprotein resulting from deamidation through the cyclic imide pathway. The separation underscores the power of protein sorting and biomimetic interaction HPLC to yield high resolution of a specific characteristic of a protein that cannot be achieved by other analytical methods.

CONCLUSIONS

The physico-chemical interactions between the stationary phase surface and the surface of a protein provides the basis of separation in chromatography. Hence, knowledge of the structure of a protein can guide the selection of the conditions, including the choice of stationary phase, to obtain a particular separation. In this report the column selection for the separation of rhDNase from its deamidated variant was guided by the insight gained from the crystal structure of bovine DNase, that revealed that deamidation occurs at a residue involved in the binding of the enzyme to DNA. Conventional ion-exchange columns, with relatively short ligands, were unable to resolve rhDNase from its deamidated variant. Columns packed with polyanionic ligands -including a tentacle cation exchanger, immobilized heparin, immobilized DNA and an immobilized synthetic DNA analogue- were able to achieve the separation of the two closely related proteins. The longer polyanionic ligands apparently can gain access to the relevant portion of the DNA binding site and mimic the interaction of DNase with its substrate and discriminate on the basis of deamidation at a single site in the glycoprotein. The physicochemical interactions involved can themselves be studied by chromatographic methods. One example of such an interaction is suggested by the separation obtained on the oligonucleotide analogue column of the isomers of the deamidated form of rhDNase, that differ only in the geometry of the acidic residue resulting from deamidation of the asparagine at residue 74. The slight structural difference between the aspartate- and isoaspartate-containing variants of deamidated rhDNase provided sufficient selectivity to permit the isolation of the individual isomeric proteins from the oligonucleotide analog column for further characterization. The chromatographic separation of the two species on the biomimetic interaction column thus sheds new light on the biochemical interaction between variants of an enyzyme and its substrate.

Both aspects of the surface interactions involved in chromatography -the column selection guided by protein structural considerations and the investigation of biochemical interactions by chromatographic methods- are examples of protein sorting, the high-resolution separation of proteins on the basis of a specific significant structural feature, without regard to the heterogeneity that may be present on the remainder of the protein. Since chromatographic resolution is essentially limited to the portion of the protein that interacts with the stationary phase, it is a powerful means of focusing the available separating power on the structural feature of interest, and sorting a population of proteins on the basis of characteristics of that feature. In this report, the structural feature of interest was Asn-74, and biomimetic interaction of the stationary phase ligands with the portion of the enzyme containing this residue was facilitated by the involvement of the residue in DNA binding. The chromatographic separation was focused on that portion of the molecule by the use of biomimetic

polyanionic ligands, and a mixture of rhDNase variants was sorted into subpopulations on the basis of the residue at the deamidation site in the protein. The subpopulations did not differ in other characteristics, including in the considerable charge heterogeneity associated with the oligosaccharides present at the two glycosylation sites on rhDNase.

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