Akira Ehara
Shinzo Nishi

Department of Biochemistry, Hokkaido University School of Medicine, Sapporo

Structural basis of electrophoretic variants of rat α-fetoprotein

The molecular basis for electrophoretic variations of rat α-fetoprotein was studied. Stepwise deglycosylations of proteins and radiolabeling of the sugars indicated that the number of such chains per molecule is two and one for the slow and fast variants, respectively.

Rat α-fetoprotein (AFP) separates into two discrete electrophoretic fractions in procedures employing starch gel [1], polyacrylamide gel in the absence [2, 3] or presence [4] of sodium dodecyl sulfate (SDS) and upon isoelectric focusing [2]. Ion exchange chromatography [5] also demonstrates such heterogeneity and it is generally accepted that the slow and fast variants have different charges [2-7] and molecular sizes [4, 8-10]. Isoelectric points determined by isoelectric focusing were pH 4.76 and pH 5.05 for the slow and fast forms, respectively [2]. Their molecular weights, determined by SDS-polyacrylamide gel electrophoresis, were 72500 and 69500, respectively [10].

The analysis of the protein portion of rat AFP by Edman degradation revealed that the two variants had identical sequences for residues 1-18 [7], but variable results were reported for the *C*-terminus. In one study, valine was found for both variants [4], while in another, glycine and valine were reported for the fast and slow variants, respectively [7]. Chemical analyses of the carbohydrate moieties of the two variants did not reveal any difference that would account for the observed heterogeneity [4, 6, 11], indicating that the heterogeneity is due to conformation or protein moiety. On the other hand, in desialidation experiments, Watanabe *et al.* [3] found that the sialic acid content of the slow variant was twice as high as the fast. Furthermore, Mano *et al.* [9] reported that carbohydrate-free rat AFP produced by hepatoma cells, cultured in the presence of tunicamycin, was electrophoretically homogeneous and had a molecular weight of only 66000. We also observed that the nonglycosylated recombinant rat AFP produced in *E. coli* was also homogeneous [10]. These data suggest that the heterogeneity of rat AFP is due to variations in its sugar components.

In the present investigation deglycosylation and radiolabeling of the sugar chains of rat AFP indicate that the slow variant has two sugar chains per molecule whereas the fast variant has only one. Previous studies of rat AFP cDNA showed it to be composed of a single peptide chain with two possible glycosylation sites, Asn-X-Thr or Asn-X-Ser, where X is any amino acid except Pro [10, 12, 13]. Our protein was isolated from the ascites fluid of rats bearing the AH66 hepatoma [2] by an immunoadsorbent method [10, 14]. The slow and fast variants were separated by ion exchange chromatography as previously described by Wong *et al.* [5]. The slow variant separated was highly homogeneous but the fast contained a trace amount of the slow (Fig. 1, lanes 3, 4).

Correspondence: Dr. Shinzo Nishi, Department of Biochemistry, Hokkaido University School of Medicine, Kita 15 Nishi 7, Kita-ku, Sapporo 060, Japan

Abbreviations: AFP, α-fetoprotein; **SDS**, sodium dodecyl sulfate

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Deglycosylation was performed with glycopeptidase F (Boehringer, Mannheim), which cleaves the linkage between asparagine and the innermost residue of the sugar chain [15]. Samples of AFP (25 μg) were denatured by heating at 100 °C for 1 min in 100 μL of 20 mM potassium phosphate, pH 7.2, 50 mM EDTA, 1 % Triton X-100, 0.2 % SDS and 144 mM 2-mercaptoethanol. These were then digested with 1 unit of enzyme at 37 °C and analyzed by SDS-polyacrylamide gel electrophoresis [16]. The molecular sizes of the AFP were found to decrease slowly and, following complete digestion, both the slow and fast variants had molecular weights of 67000. Nonglycosylated rat AFP, which was produced by the AH66 cells in the presence of tunicamycin by the method of Mano *et al.* [9], has the same molecular weight (data not shown). Partial digestion of the slow variant produces a component of molecular weight of 69500, which is identical to that of the fast variant. The sugar chains of rat AFP have molecular weights of 2224 and 2427 [11]. The differences in molecular sizes of the fast, slow, and deglycosylated AFP suggest the slow variant having 2 and the fast variant 1 sugar chain.

To further establish this stoichiometry, rat AFP was labeled with [^{3}H]mannose and the specific activities of the slow and fast AFP were compared. Such an approach is feasible since Bayard *et al.* [11] reported that there are two kinds of sugar

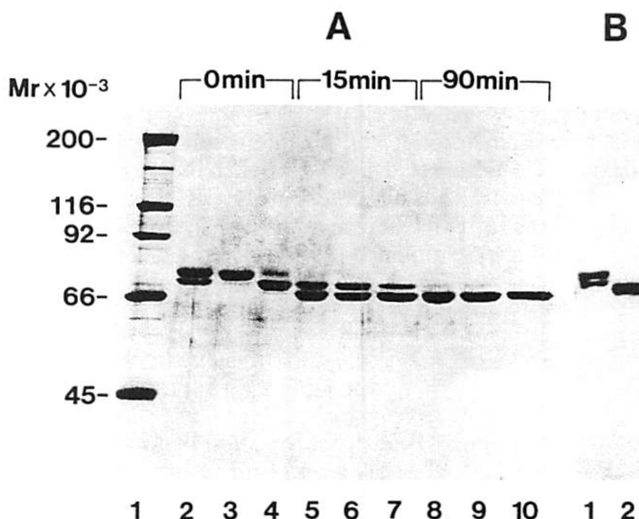

Figure 1. SDS-polyacrylamide gel electrophoresis of rat AFP in an 8 % polyacrylamide gel, followed by staining with Coomassie Brilliant Blue. (A) AFP (lanes 2, 5, 8), slow variant (lanes 3, 6, 9) and fast variant (lanes 4, 7, 10). Each of the three samples shows results after 0, 15, and 90 min, respectively, of digestion with glycopeptidase F. Molecular size markers (lane 1) were myosin (M_r 200000), β-galactosidase (M_r 116300), phosphorylase b (M_r 92500), bovine serum albumin (M_r 66200) and ovalbumin (M_r 45600). (B) Radiolabeled AFP electrophoresed before (lane 1) and after (lane 2) digestion with glycopeptidase F.

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chains on rat AFP. The two structures are similar, except that one has an additional bisecting N-acetyl-D-glucosamine residue. AH66 cells (7×10^6) were collected from ascitic fluid of innoculated rats and incubated at 37 °C for 48 h with 11 μCi/mL of [^{3}H]mannose (30 Ci/mmol, Amersham) in a 25-cm^2 flask containing 7 mL of Dulbecco's modified Eagle's medium supplemented with 10 % fetal calf serum. The radiolabeled AFP was purified from the spent culture medium as described above and 2 μg of unmodified and deglycosylated protein were subjected to SDS-polyacrylamide gel electrophoresis. The amounts of slow and fast variants determined by densitometry of the stained gel were 1.3 μg and 0.7 μg, respectively. Gel slices containing these components were excised, dissolved in 0.5 mL of H_2O_2 at 50 °C, and their radioactivities measured. The specific radioactivities of slow and fast variants were 548 dpm/μg and 298 dpm/μg, respectively, and that of deglycosylated AFP was 65 dpm/μg. The low radioactivity of deglycosylated AFP is considered to be due to the nonspecific binding of the radioactive mannose since the molecular size observed obviates it being glycosylated protein. The specific activities of the slow and fast variants calculated by correcting for the nonspecific binding are 483 dpm/μg and 233 dpm/μg, respectively, and the ratio is 2.07.

From the results of deglycosylation and the radiolabeling experiments it may be concluded that the slow variant of rat AFP has two and the fast variant has one sugar chain. Watanabe *et al.* [3] reported that upon digestion with neuraminidase, the slow and fast variants were converted to slower forms through two and one intermediate product, respectively. Since two sialic acid residues were reported to be present on one sugar chain [11], their observation supports our conclusion. However, this does not agree with several previous reports, Kerckaert *et al.* [4, 6] measured sugar contents of the two variants isolated and obtained essentially the same values for them. Furthermore, their group reported that rat AFP had two sugar chains per molecule [11]. The assumption that slow and fast variants, carrying two sugar chains that differ slightly, generate stable conformational isomers, which show different apparent molecular sizes (as observed in electrophoresis), is not likely because the fast variant had a specific activity that was clearly lower than that of the slow variant in radiolabeling, and no intermediate product was detected upon its partial deglycosylation. It is noteworthy that human AFP, which has one potential glycosylation site [17] is homogeneous [8, 18], whereas mouse AFP, which has three potential glycosylation sites [19], is heterogeneous in electrophoresis [8, 18]. We estimated the potential glycosylation sites of rat AFP to be two because one of the three potential glycosylation sites (as described in [17]) has the sequence Asn-Pro-Ser, which is not glycosylated [20]. The source of AFP used by Kerckaert *et al.* [4, 6, 11] was rat amniotic fluid and the source in the present study was of hepatoma origin, although this hardly reconciles the discrepancies described above.

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