Biotin binding to avidin

Oligosaccharide side chain not required for ligand association

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A commercially available, purified preparation of avidin was found to comprise two polypeptide bands (M_r 18000 and M_r 15500 respectively). Both bands bound biotin as assessed by biotin overlays of protein blots. The M_r 15500 polypeptide was found to differ from the M_r 18000 polypeptide only in its sugar content. When the commercial preparation was applied to a concanavalin A affinity column, the glycosylated forms were retarded as expected, and homotypic nonglycosylated avidin tetramers which failed to bind selectively to the column were collected in the effluent. The biotin-binding properties of the nonglycosylated avidin were equivalent to those obtained for the native (glycosylated) avidin molecule, indicating that the oligosaccharide moiety is not essential for the binding activity.

INTRODUCTION

Egg white avidin is a basic glycoprotein having a molecular mass of about 67000 and an isoelectric point of 10.5 (Green, 1975). Extensive interest in avidin has persisted, mainly due to its unusually strong interaction with the vitamin biotin. The complex formed between these two biomolecules has a remarkably low dissociation constant ($K_D \sim 10^{-15}$ M; Green, 1963). Because of this tenacious interaction, the avidin–biotin system has proved a powerful tool in molecular biology (Bayer & Wilchek, 1980) and in many immunological systems (Wilchek & Bayer, 1984) for a variety of research, clinical and industrial purposes. However, precise knowledge regarding the molecular mechanisms responsible for such tight binding has yet to be discovered.

Avidin contains four essentially identical subunits, each of which consists of a single polypeptide chain bearing 128 amino acids and possessing one biotin-binding site. The amino acid composition and the complete sequence of avidin have been established by DeLange & Huang (1971).

The carbohydrate moiety (about 10% of the total molecular mass), which reportedly contains four or five residues of mannose and three residues of *N*-acetylglucosamine per subunit, is *N*-linked to asparagine residue 17 (DeLange, 1970). The composition and structural arrangement of the oligosaccharide residues on avidin have been shown to exhibit a high degree of heterogeneity (Bruch & White, 1982).

In this communication we report the isolation of nonglycosylated avidin and demonstrate that the oligosaccharide side chain is not required for biotin binding.

EXPERIMENTAL

Materials

Egg white avidin was obtained from two sources; Belovo avidin (batch 1283) was provided by Soc. Coop.

Belovo (Bastogne, Belgium), and Sigma avidin (lot no. 96C-9540) was purchased from Sigma. The following materials were also obtained from Sigma: alkaline phosphatase (type VII-NT), naphthol AS-MX phosphate (free acid), Fast Red TR salt, bovine serum albumin, horseradish peroxidase, and 3,3'-diaminobenzidine tetrahydrochloride. Concanavalin A (Con A) and wheat germ agglutinin conjugated to horseradish peroxidase were obtained from Bio-Yeda (Rehovot, Israel). Other chemicals were of the purest analytical grade available. Biotinylated alkaline phosphatase was prepared using biotinyl *N*-hydroxysuccinimide ester according to published procedures (Bayer *et al.*, 1986).

Slab gel electrophoresis and blotting

SDS / polyacrylamide - gel electrophoresis (SDS/PAGE) was performed using the discontinuous buffer system of Laemmli (1970). Electrophoresis was carried out for 18 h at a constant current of 7 mA. Cathodic discontinuous slab PAGE system (pH 4.5) was performed according to Reisfeld *et al.* (1962). Following electrophoresis, gels were removed from the mould, and portions were either stained with Coomassie Brilliant Blue R or subjected to blotting. Blotting onto nitrocellulose membrane filters was carried out in a gradient electric field as previously described (Gershoni & Palade, 1982; Gershoni *et al.*, 1985b).

Biotin-binding activity on blots

All stages described here were performed at room temperature with shaking. Strips or sheets of nitrocellulose membrane which contained the protein(s) were quenched for 1 h in 2% bovine serum albumin in 10 mM-phosphate-buffered saline, pH 7.4. The nitrocellulose strips were rinsed with phosphate-buffered saline and incubated for 1 h with biotinyl alkaline phosphatase (1–3 µg/ml in 2% bovine serum albumin). The blots were washed three times with phosphate-buffered saline and alkaline phosphatase substrate solution (containing

Abbreviations used: Con A, concanavalin A; α MM, methyl α -D-mannoside; PAGE, polyacrylamide-gel electrophoresis.

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0.01% naphthol AS-MX phosphate and 0.03% Fast Red TR salt in 0.1 M-Tris/HCl buffer, pH 8.4) was added. The signal associated with avidin tetramers appeared within 30 min, whereas that of the monomers appeared within 2 to 3 h and progressively increased over a 24 h incubation period. The enzymic reaction was terminated by exchanging the substrate solution with double-distilled water.

Biotin-binding activity in solution

Biotin-binding capacity of avidin preparations in solution was determined using biotinyl bovine-serum-albumin-coated microtitre plates as reported earlier (Bayer *et al.*, 1986). Various dilutions of the given avidin preparations (1.3, 2, 3, 4.5, 7 and 10 μ g/ml) were applied to the plates, and subsequent interaction with biotinyl alkaline phosphatase was assessed. Tests were performed in triplicate, and all incubations were carried out at room temperature. Plates were read repeatedly at various time intervals; the slopes of the resultant curves were compared for a given concentration of the avidin samples.

Sugar analyses

A variety of approaches were employed for the analysis of carbohydrates. For general detection of sugars, blots were oxidized with NaIO_4 and subsequently stained with alkaline phosphatase hydrazide (Gershoni *et al.*, 1985a). Mannose was demonstrated using the two-step Con A-peroxidase method (Clegg, 1982). The presence of *N*-acetylglucosamine residues was determined using wheat germ agglutinin conjugated to horseradish peroxidase. In both cases, after incubation with the lectins the blots were washed with phosphate-buffered saline and immersed in a solution containing 0.03% diaminobenzidine and 0.02% H_2O_2 in 50 mM-Tris/HCl buffer, pH 7.4, in the presence of 200 mM-NaCl. Total sugar content of the avidin preparations was also monitored by g.l.c., whereby trimethylsilyl ether derivatives were detected on an SE-30 column. Hexosamines were liberated by hydrolysis in 4 M-HCl at 100 °C for 4 h under nitrogen (Marshall & Neuberger, 1972), and the neutralized hydrolysates were analysed by the Morgan-Elson method using glucosamine as standard (Rondle & Morgan, 1955).

Affinity chromatography on Con A columns

Separation of the nonglycosylated avidin tetramers from the glycosylated forms of the protein was achieved using Con A-agarose columns. Two preparations [Con A-agarose from Makor Chemicals, Jerusalem (7.5 mg/ml) and Glycosylex A from Bio-Yeda, Rehovot, Israel (10.4 mg/ml)] were used interchangeably and gave similar results.

The affinity column was first equilibrated with buffer which contained 25 mM-Tris/HCl (pH 7.4), 150 mM-NaCl, 1 mM- CaCl_2 and 1 mM- MgCl_2 . A solution of Belovo avidin, dissolved either in distilled water or the same buffer (as described later) was centrifuged (at 4000 rev./min for 10 min) in a Sigma 3E-1 laboratory centrifuge (Osterode, West Germany). The clarified solution was loaded on the Con A column, and the column was washed with the buffer. The effluent fractions were first examined spectrophotometrically and then by SDS/PAGE to ensure that only those containing the nonglycosylated forms were pooled. The bound glycosylated forms were eluted using a 5% solution of methyl α -D-mannoside (α MM) dissolved in the same buffer.

Miscellaneous methods

Amino acid analysis was performed on a Dionex D-500 amino acid analyser. Protein samples were hydrolysed with 6 M-HCl at 110 °C for 20 h in vacuum-sealed Pyrex tubes. Hydrazinolysis was employed for C-terminal detection (Fraenkel-Conrat & Tsung, 1967). Protein samples (1 mg/ml) were hydrolysed with an excess of hydrazine (200 μ l treated with nitrogen gas) at 100 °C for 5 h in vacuum-sealed Pyrex tubes. The residual C-terminal amino acid was detected by amino acid analysis. *N*-Terminal sequencing was performed using a model 470 A Protein Peptide Sequencer from Applied Biosystems Inc.

RESULTS

Properties of commercial preparations of avidin

Two commercial preparations of avidin (Belovo and Sigma) were subjected to SDS/PAGE, blotted and probed with biotinyl alkaline phosphatase as shown in Fig. 1. Although it has previously been reported (Green, 1975) that the four subunits of avidin are identical, we were able to resolve two subunits from Belovo avidin (molecular masses about 18000 and 15500). The Sigma avidin gave only one subunit (molecular mass 18000). The appearance of dimers of Sigma avidin was more pronounced than that observed for Belovo avidin. The upper Belovo band migrated slightly faster than the Sigma subunit which may be due to the heterogeneity of the respective oligosaccharide moieties. Both subunits of Belovo, as well as the Sigma avidin subunit, were able to bind biotinyl alkaline phosphatase (Fig. 1).

Biotin binding of the two subunits was determined in the presence or absence of β -mercaptoethanol and after incubation for 10 min either at room temperature or at 100 °C for 5 h in vacuum-sealed Pyrex tubes. The residual activity affected the capacity of the subunits of Belovo and Sigma avidin to bind biotin (results not shown).

The binding of biotinyl alkaline phosphatase to avidin subunits on blots appears to be specific, since excess biotin prevented the binding, and other polypeptides were not labelled extensively using biotinyl alkaline phosphatase (Fig. 2). In addition, underivatized alkaline phosphatase failed to bind avidin.

A wide range of avidin concentrations was checked for biotin-binding activity. The intensity of the binding was concentration-dependent and the activity associated with the subunits could not be detected below avidin concentrations of 10 μ g. Optimal results were achieved when the blots were probed with 1–3 μ g of biotinyl alkaline phosphatase/ml. The binding was stable; after 24 h of incubation at 25 °C in phosphate-buffered saline, no appreciable reduction in the signal could be detected.

Belovo avidin was particularly interesting because of the lower-molecular-mass subunit. Therefore the molecular nature of the difference(s) between the two subunits was examined. Amino acid analysis of Belovo and Sigma avidins revealed identical amino acid compositions. The *N*-terminal residues of both avidin preparations were determined, and the sequence of Ala-Arg-Lys obtained was identical to that of the published sequence (DeLange & Huang, 1971). *C*-Terminal analysis revealed only one amino acid (glutamic acid) for avidin preparations from both commercial sources. Consequently it was concluded that both Belovo subunits and the Sigma preparation

Fig. 1. Electrophoretic mobility and biotin-binding capacities of Belovo and Sigma avidin

Belovo avidin (lanes A and C) and Sigma avidin (lanes B and D) ($30 \mu\text{g}$ each) were resolved on 10% SDS/PAGE. The gels were then either stained with Coomassie Brilliant Blue (lanes A and B) or blotted on nitrocellulose membrane filters (lanes C and D). Blots were then probed with biotinyl alkaline phosphatase ($3 \mu\text{g}/\text{ml}$) and examined for phosphatase activity. The activity signal appeared after 2–3 h. The above are representative results of such experiments.

had identical amino acid sequences. Sugar analyses were therefore conducted to ascertain whether or not the observed difference in molecular mass reflects differential glycosylation for the two subunits. Indeed, preliminary experiments involving enzyme hydrazide- and lectin-mediated staining of blots indicated that the high-molecular-mass subunits in Belovo and Sigma avidins are glycosylated whereas the low-molecular-mass subunit of Belovo avidin is not (as will be discussed below).

Isolation of nonglycosylated avidin

In view of the above it was thought that a purified nonglycosylated avidin fraction could be obtained by passing Belovo avidin through a Con A column. Indeed, when Belovo avidin, dissolved in buffer, is applied to such a column, nonglycosylated avidin appears in the effluent and can be separated from glycosylated forms which adhere to the column (Fig. 3a). It was found that the procedure can be improved by dissolving Belovo avidin in distilled water rather than buffer before applying the sample to the column. In this case, all types of avidin (including nonglycosylated avidin) were adsorbed onto the column (Fig. 3b). Selective release of the nonglyco-

Fig. 2. Specificity of biotin-binding assay on blots

Samples of lysozyme (lanes A, D, G and J), Belovo avidin (lanes B, E, H and K), and membranes of *Torpedo californica* electric organ (lanes C, F, I and L) ($30 \mu\text{g}$ each) were resolved on 10% polyacrylamide gels. Gels were either stained with Coomassie Brilliant Blue (lanes A–C) or blotted onto nitrocellulose membrane filters (lanes D–L). Blots were then probed with $3 \mu\text{g}/\text{ml}$ of biotinyl alkaline phosphatase (BAP) (lanes D–F), with $3 \mu\text{g}/\text{ml}$ of biotinyl alkaline phosphatase in the presence of $100 \mu\text{g}/\text{ml}$ of free biotin (BAP + B) (lanes G–I), or with underivatized alkaline phosphatase (AP) ($7.3 \mu\text{g}/\text{ml}$) (lanes J–L). Note that the high background in lanes J–L is due to the excess alkaline phosphatase used. Lysozyme, a positively charged protein, appears to give relatively low levels of nonspecific reaction in all three blots.

sylated avidin could be achieved by increasing the ionic strength of the wash (i.e. by washing the column in an appropriate buffer).

This phenomenon could be exploited for the purification of large quantities of nonglycosylated avidin. Application of relatively large volumes of dilute protein in water ($0.5 \text{ mg}/\text{ml}$; higher concentrations gave turbid solutions) allowed accumulation of nonglycosylated avidin on the column.

Thus, preparative amounts of Belovo avidin (87 mg dissolved in 192 ml of distilled water) were loaded onto a Con A column (25 ml). Following application, a buffered solution (25 mM-Tris/HCl, pH 7.4, which contained 150 mM-NaCl, 1 mM- CaCl_2 and 1 mM- MgCl_2) was used to wash the column, after which the nonglycosylated avidin peak appeared. Elution of the adsorbed glycosylated forms was accomplished with 5% αMM in the same buffer. The affinity chromatographic pattern is shown in Fig. 4. The fractions which contained nonglycosylated avidin (shaded area of Fig. 4), and those which were eluted with the competing sugar, were pooled separately; the two preparations were dialysed exhaustively and lyophilized. The yield of nonglycosylated avidin was 24.7 mg (30%). The amount of the glycosylated forms of avidin which could be recovered by αMM elution was nearly quantitative (54 mg, 65%).

Characterization of nonglycosylated avidin

The purified nonglycosylated avidin preparation was characterized on SDS/PAGE by only one band, which

Fig. 3. Affinity chromatographic pattern of Belovo avidin on Con A-agarose column

The abscissa denotes the washings upon application and percolation of the sample through the column (see text for details). The various events (i.e. application of sample, substitution of buffer for distilled water in effluent fractions, and elution of glycosylated avidin using α MM) are designated in the Figure by arrows. (a) A solution of Belovo avidin (2 mg in 2 ml of buffer) was applied to a 2 ml Con A column. Note that a protein peak appears immediately in the effluent, whereas a second peak can be selectively released with α MM. (b) When a similar solution of Belovo avidin, dissolved in distilled water rather than buffer, is applied to the same column, no peak appears in the effluent until buffer is added. In both cases (a and b) the first peak was found to be nonglycosylated avidin.

migrated at a position coincident with that of the low-molecular-mass band of Belovo avidin (Fig. 5). The α MM-eluted glycosylated form of avidin consisted of two bands, similar to the originally applied sample, except that the lower band appeared somewhat fainter. Carbohydrate analysis of the avidin polypeptides substantiated that nonglycosylated avidin was essentially devoid of sugars (Fig. 5). These results were confirmed by gas chromatography.

C-Terminal, N-terminal and amino acid analyses indicated that the purified nonglycosylated avidin constitutes the intact polypeptide. Using cathodic discontinuous PAGE, the nonglycosylated avidin preparation migrated as a tetramer at a position slightly faster than that of the major band associated with the original Belovo preparation. The Sigma preparation migrated significantly slower than nonglycosylated avidin, reflective of the elevated levels of oligosaccharide in the former (data not shown).

Fig. 4. Preparative purification of nonglycosylated avidin by chromatography on Con A-agarose

A solution of Belovo avidin (approx. 0.5 mg/ml of distilled water) was applied to a 25 ml Con A-agarose column until protein appeared in the effluent (thus indicating saturation of the column). The nonglycosylated avidin was eluted with buffer. The glycosylated forms of avidin were eluted with three sequential volumes (about 30 ml each) of buffer containing α MM.

Fig. 5. Carbohydrate-specific staining of blotted avidin preparations

Samples (15 μ g each) of Belovo avidin (lanes A), Sigma avidin (lanes B), affinity-purified nonglycosylated avidin (lanes C) and the α MM-eluted glycosylated forms of the protein (lanes D) were resolved on 12% SDS/PAGE. Gels were either stained with Coomassie Brilliant Blue (CBB) or blotted onto nitrocellulose membrane filters. Blots were then probed successively with Con A (10 μ g/ml) and horseradish peroxidase (10 μ g/ml) or with peroxidase-conjugated wheat-germ agglutinin (WGA) (1.5 μ g/ml) and examined for peroxidase activity.

The nonglycosylated avidin preparation was capable of binding biotinyl alkaline phosphatase following SDS/PAGE or cathodic discontinuous PAGE. The biotin-binding capacity of nonglycosylated avidin was compared quantitatively to those of the commercial prep-

Table 1. Relative activity of the different avidin preparations

Equivalent concentrations of the respective avidin preparations were examined for biotin-binding activity in solution. In each experiment, the biotin-binding activity observed for Sigma avidin was arbitrarily taken as 1.0.

Avidin	Relative activity
Sigma	1.0
Belovo	0.98 ± 0.01
Nonglycosylated	0.94 ± 0.02
αMM-eluted	0.94 ± 0.03

arations by enzyme assay on microtitre plates which contained biotinylated bovine serum albumin (Bayer *et al.*, 1986). Using this procedure, essentially no differences were recorded among the various avidin preparations tested (Table 1).

DISCUSSION

Upon examining different commercial preparations of avidin, it was found that avidin obtained from Belovo was characterized by two bands on SDS/PAGE whereas that from Sigma had only one band. Initially, it was thought that the extraneous band in the former represented a lower-molecular-mass contaminant (perhaps lysozyme). However, by ligand overlay of protein blots it was clear that both bands of Belovo avidin were equally capable of binding biotin.

The overlay assay developed in this work allows the qualitative detection of biotin-binding proteins. However, the fact that relatively high amounts (10 µg) of avidin are required for the demonstration of biotin-binding on blots indicates that the ligand binding is considerably less efficient than anticipated. At present, one cannot distinguish between at least two possible explanations for this: either the affinity of the avidin monomer is less than that of the tetramer or some reassociation of subunits occurs during the blotting procedure, thereby rendering low amounts of active tetramers. The question regarding the activity of avidin monomers has been of concern in previous studies as well (Green & Toms, 1973).

The sole difference between the high- and low-molecular-mass subunits in commercial avidin preparations obtained from Belovo is reflected in the respective carbohydrate content of these bands. It is still not clear, however, whether the observed differences in glycosylation are artifactual and result from deglycosylation during purification procedures or whether they reflect actual biological differences, such as sub-species variations in the source of chicken populations used.

Nonetheless, by exploiting this situation we have been able to test the requirement for the sugar residues in the biotin-binding process. It is clear from our results that nonglycosylated avidin tetramers are as efficient as the

glycosylated forms in binding biotin. This conclusion is further supported by the fact that the bacterial biotin-binding protein, streptavidin, is also devoid of sugars (Chalet & Wolf, 1964).

The demonstration that nonglycosylated avidin can be purified might also have practical applications since the carbohydrate moieties of avidin might increase non-specific association in various diagnostic assays in which avidin-biotin technology is employed (particularly if sugar-binding proteins are present). The availability of two kinds of avidin (glycosylated and nonglycosylated) is important for future studies of the function of the sugar moieties. The oligosaccharide side chains may have an effect on the secondary, tertiary or quaternary structure of avidin. They may also be involved in the uptake of avidin by cell surface mannose receptors (e.g. as a storage protein for biotin) or by subcellular organelles (such as lysosomes). The availability of the native (glycosylated) and nonglycosylated proteins may also be helpful in the general understanding of the biological role of carbohydrates of glycoproteins.

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