Biochem. J. (1991) 278, 573–585 (Printed in Great Britain)

Studies on the biotin-binding site of avidin

Minimized fragments that bind biotin

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The object of this study was to define minimized biotin-binding fragments, or 'prorecognition sites', of either the egg-white glycoprotein avidin or its bacterial analogue streptavidin. Because of the extreme stability to enzymic hydrolysis, fragments of avidin were prepared by chemical means and examined for their individual biotin-binding capacity. Treatment of avidin with hydroxylamine was shown to result in new cleavage sites in addition to the known Asn–Gly cleavage site (position 88–89 in avidin). Notably, the Asn–Glu and Asp–Lys peptide bonds (positions 42–43 and 57–58 respectively) were readily cleaved; in addition, lesser levels of hydrolysis of the Gln–Pro (61–62) and Asn–Asp (12–13 and 104–105) bonds could be detected. The smallest biotin-binding peptide fragment, derived from hydroxylamine cleavage of either native or non-glycosylated avidin, was identified to comprise residues 1–42. CNBr cleavage resulted in a 78-amino acid-residue fragment (residues 19–96) that still retained activity. The data ascribe an important biotin-binding function to the overlapping region (residues 19–42) of avidin, which bears the single tyrosine moiety. This contention was corroborated by synthesizing a tridecapeptide corresponding to residues 26–38 of avidin; this peptide was shown to recognize biotin. Streptavidin was not susceptible to either enzymic or chemical cleavage methods used in this work. The approach taken in this study enabled the experimental distinction between the chemical and structural elements of the binding site. The capacity to assign biotin-binding activity to the tyrosine-containing domain of avidin underscores its primary chemical contribution to the binding of biotin by avidin.

INTRODUCTION

The egg-white glycoprotein avidin and the bacterial protein streptavidin exhibit similar exceptionally high affinities (K_a approx. 10^{15} M^{-1}) for the vitamin biotin (Green, 1975, 1990). Despite the apparent lack of immunochemical and evolutionary relatedness, both proteins share many structural properties. Both are highly stable tetramers (M_r approx. 66000) with a single biotin-binding site per subunit. The primary structures are characterized by a remarkable conservation in sequence, which is largely confined to relatively short homologous stretches that form defined domain-like segments (Argarana *et al.*, 1986; Wilchek & Bayer, 1989; Bayer & Wilchek, 1990*a*). Tryptophan and tyrosine residues have been consistently implicated in the biotin-binding sites of both proteins (Green, 1975; Gitlin *et al.* 1988*a*,*b*, 1990; Weber *et al.*, 1989; Kurzban *et al.*, 1989, 1990).

Early immobilization studies (Green & Toms, 1973; Henrikson *et al.*, 1979; Kohanski & Lane, 1990) suggested that individual subunits (monomers) of avidin could bind biotin, albeit at markedly lower affinity (approx. 10^7 M^{-1}). These results appeared to have been confirmed by blotting of SDS/PAGE-separated subunits of avidin and streptavidin (Hiller *et al.*, 1987).

In both cases the size of the avidin and streptavidin monomers capable of binding biotin could be effectively decreased. In this context, non-glycosylated subunits of avidin bound biotin, similarly to the glycosylated form (Hiller *et al.*, 1987), also indicating the irrelevance of the carbohydrate moiety to biotin binding. Streptavidin, on the other hand, is subject to chemical and proteolytic truncation of extraneous *N*- and *C*-terminal appendages (Argarana *et al.*, 1986; Bayer *et al.*, 1986, 1989; Pähler *et al.*, 1987), and the resultant 'core' protein binds free biotin with a tenacity equal to that of the native unprocessed molecule.

In the present study, we address the question of whether the size of either the avidin or streptavidin monomer can be further decreased with retention of the biotin-binding property by one or more of its fragments. We describe chemical methods for cleavage of the avidin molecule at different defined positions to produce such fragments. We considered that, if we begin with the high-affinity constant of the avidin-biotin constant, we would still be able to detect the decreased levels of binding that would be expected in the active fragment(s). Indeed, the biotin-binding affinities of such fragments were much lower than that of the intact monomer, and, in order to demonstrate binding, novel ultrasensitive biotin-binding assays were developed.

MATERIALS AND METHODS

Materials

Egg-white avidin was obtained from Belovo Chemicals (Bastogne, Belgium), STC Laboratories (Winnipeg, Manitoba, Canada) or Sigma Chemical Co. (St. Louis, MO, U.S.A.). The following materials were also obtained from Sigma Chemical Co.: (+)-biotin, 3-(cyclohexylamino)-1-propanesulphonic acid (Caps), 2-mercaptoethanol, bovine pancreas carboxypeptidase A treated with phenylmethanesulphonyl fluoride (50 units/mg of protein), pig pancreas carboxypeptidase B treated with di-isopropyl phosphorofluoridate (100 units/mg of protein), carboxypeptidase Y from baker's yeast (100 units/mg of protein), BSA, horseradish peroxidase, 3,3′-diaminobenzidine tetrahydrochloride and standard kits of M_r markers.

The medium-range-M_r kit (Sigma catalogue no. MW-SDS-70L) contained the following proteins: BSA (M_r 66000), ovalbumin (M_r 45000), glyceraldehyde-3-phosphate dehydrogenase (M_r 36000), carbonic anhydrase (M_r 29000), trypsinogen (M_r 24000), soya-bean trypsin inhibitor (M_r 20100) and α-lactalbumin (M_r 14200). The low-M_r kit (Sigma catalogue no. MW-SDS-17) contained the following: myoglobin polypeptide backbone (M_r 16950), myoglobin fragments I+II (M_r 14400), myo-

Abbreviations used: B-BSA, biotinylated BSA; PVDF, poly(vinylidene difluoride); Caps, 3-(cyclohexylamino)-1-propanesulphonic acid.

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globin fragment I (M_r 8160), myoglobin fragment II (M_r 6210) and myoglobin fragment III (M_r 2510).

Proline-specific endopeptidase (EC 3.4.21.26) was obtained from Seikagaku Kogyo Co. (Tokyo, Japan). All other proteinases used in this work were from Sigma Chemical Co. Concanavalin A was obtained from Bio-Makor (Rehovot, Israel).

Formic acid, CNBr, trichloroacetic acid and chloramine-T (*N*-chlorotoluene-*p*-sulphonamide sodium salt) were obtained from Fluka A.G. (Buchs, Switzerland). Hydrazine (anhydrous) was purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.) and Amido Black (Buffalo Black NBR) from Allied Chemicals Industrial Chemicals Division (Morristown, NJ, U.S.A.). Hydroxylammonium chloride, SDS, ammonium persulphate, glycine, glycerol and sodium metabisulphite were purchased from BDH Chemicals (Poole, Dorset, U.K.). Acrylamide and KI were obtained from Merck (Darmstadt, Germany). [*carbonyl*-^{14}C]Biotin and Na^{125}I in NaOH solution (100 mCi/ml) were obtained from Amersham International (Amersham, Bucks., U.K.).

Sephadex G-75 was from Pharmacia Chemical Co. (Uppsala, Sweden). Cellulose dialysis tubing of appropriate M_r cut-off was obtained from Spectrum Medical Industries (Los Angeles, CA, U.S.A.). Cellulose nitrate membrane filters (BA 85; pore size 0.45 μm) were obtained from Schleicher und Schüll (Dassel, Germany) and poly(vinylidene difluoride) (PVDF) membranes (pore size 0.45 μm) from Millipore Corp. (Bedford, MA, U.S.A.). Falcon 96-well flexible assay plates were purchased from Becton Dickinson (Oxnard, CA, U.S.A.). All other chemicals were of the purest analytical grade available.

Control peptide A, used in the competition assay, comprised a synthetic 15-residue sequence of the β-subunit of cholera toxin (generously provided by Dr. Reuben Hiller, Department of Polymer Research at our Institute). Control peptide B was kindly donated by Dr. Rachel Mosckovitz of our Department and represented as a synthetic 17-residue sequence derived from the α-subunit of the acetylcholine receptor from *Torpedo californica*.

Streptavidin preparations

Streptavidin was prepared in our laboratory from the spent growth medium of *Streptomyces avidinii* with the use of an improved iminobiotin–Sepharose affinity column as described previously (Bayer *et al.*, 1986). Truncated forms of the protein were prepared by subjecting purified samples to extracts of the culture medium or to purified proteinases (Bayer *et al.*, 1989). Quantities of core streptavidin were also generously supplied by Boehringer Mannheim G.m.b.H. (Mannheim, Germany).

Proteolytic treatment of proteins

To 100 μg samples of avidin or streptavidin dissolved in 90 μl of an appropriate buffer was added a solution (10 μl) containing 2 μg of the desired proteinase. The following buffer systems were used for the given proteinases: 0.1 M-sodium bicarbonate buffer, pH 8, for trypsin and chymotrypsin; 0.1 M-sodium acetate buffer, pH 5, for thermolysin; 0.1 M-sodium phosphate buffer, pH 7.5, for subtilisin and *Streptomyces griseus* proteinase; 5 mM-HCl for pepsin; 0.1 M-Tris/HCl buffer, pH 8.8, for elastase; 0.1 M-sodium phosphate buffer, pH 6.2, containing 1 mM-EDTA and 5 mM-cysteine for papain; 0.1 M-sodium phosphate buffer, pH 7.0, for proline-specific endopeptidase; 0.125 M-Tris/HCl buffer, pH 6.8, containing 0.5% SDS and 1 mM-EDTA for proteinase from *Staphylococcus aureus* strain V8; 10 mM-Tris/HCl buffer, pH 7.8, containing 5 mM-EDTA and 0.5% SDS for proteinase K. The reactions were allowed to take place at 37 °C (except for pepsin and papain, which were incubated at 25 °C). After 15 min and 2 h (unless otherwise indicated in the text) 50 μl portions were removed and combined with 25 μl of sample buffer (for composition see under 'SDS/PAGE' below). The solution was boiled for 10 min and stored at −20 °C pending SDS/PAGE analysis. The activity of each of the proteinases was confirmed in a separate experiment with BSA as a model substrate.

Hydroxylamine treatment

For qualitative work, avidin and non-glycosylated avidin were treated with 2 M-hydroxylamine in 0.2 M-Tris/HCl buffer, pH 9 (hydroxylamine/Tris buffer), as described by Bornstein & Balian (1977). The protein sample dissolved in hydroxylamine/Tris buffer (1 mg/ml) was subjected to hydrolysis at 45 °C in an oil bath. Portions (50 μl) were removed at the indicated intervals, combined with 25 μl of sample buffer, boiled for 10 min and subjected to SDS/PAGE.

For larger preparations, a solution (5 mg/ml) of avidin or non-glycosylated avidin was treated with hydroxylamine/Tris buffer at 45 °C with stirring for 6 days. During the hydrolysis, a white precipitate was obtained. The reaction mixture (including the precipitate) was dialysed against double-distilled water and stored in small portions at −20 °C pending SDS/PAGE analysis and activity measurements.

CNBr cleavage

In the presence of formic acid. Avidin or non-glycosylated avidin samples were treated with an excess of CNBr (at least 80 mol/mol of methionine) in the presence of 70% (v/v) formic acid as described by Gross & Witkop (1962) as modified by Steers *et al.* (1965). In a typical experiment 10 mg of protein in 100 μl of double-distilled water was treated with 17 mg of CNBr dissolved in 240 μl of 98% (v/v) formic acid. The reaction was carried out for 20 h at room temperature in the dark. The reaction mixture was then diluted with double-distilled water (5 ml), frozen with liquid air and kept in a desiccator containing NaOH under high vacuum. The resultant oily residue was suspended in double-distilled water and evaporated again several times, until a powdered product was obtained.

The CNBr-cleaved avidin preparation was dissolved in 1–2 ml of a solution containing 30% (v/v) acetic acid and formic acid in a 1:1 ratio and applied to a column (1.0 cm × 90 cm) of Sephadex G-75 (fine grade) pre-equilibrated with 30% (v/v) acetic acid. Elution was performed with 30% (v/v) acetic acid at a flow rate of 12.5 ml/h, and 1.3 ml fractions were collected. The absorbance of the effluent fractions was monitored at 280 nm. The column was washed with the same buffer until no further protein was eluted. Portions of peak fractions were evaporated and subjected to SDS/PAGE.

In the presence of HCl. Avidin and non-glycosylated avidin were treated with CNBr in the presence of 0.1 M-HCl (Spande *et al.*, 1970). To the protein samples (5 mg/ml in 0.1 M-HCl) was added an excess of CNBr (dissolved in acetonitrile) (at least 80 mol/mol of methionine). In a typical procedure 10 mg of protein in 2 ml of 0.1 M-HCl was treated with 13 mg of CNBr dissolved in 70 μl of acetonitrile. The hydrolysis was carried out at room temperature in the dark with stirring for 1 or 4 days. The reaction mixture was then treated in the same manner as described above.

Estimation of extent of CNBr cleavage. The extent of cleavage at the relevant methionine residue of avidin was calculated algebraically from densitometry tracings of SDS/PAGE profiles of the appropriate CNBr-treated material. It is noted (Smith, 1990) that such calculations are limited in accuracy, particularly since the expected band for residues 1–18 was not detected and the amount of the CN3 band was not equivalent to the sum of the CN2a and CN2b bands. Nevertheless, the information gives a general indication of the susceptibility of the designated bonds to CNBr cleavage.

Sequential treatment with CNBr and hydroxylamine

Portions of glycosylated avidin and non-glycosylated avidin derivatives obtained by CNBr cleavage in the presence of 0.1 M-HCl were freeze-dried and subjected to hydrolysis with hydroxylamine. In a typical experiment 2.5 mg of the CNBr-treated protein was hydrolysed with 1 ml of hydroxylamine/Tris buffer. The reaction was carried out for 6 days at 45 °C in an oil bath with stirring as described above. Portions were removed at the indicated intervals, subjected to SDS/PAGE and assayed for biotin-binding activity.

The reversed sequential treatment of avidin was also performed, whereby the hydroxylamine-cleaved preparation was subsequently subjected to CNBr treatment. Portions of glycosylated and non-glycosylated avidin derivatives obtained after 6 days of hydrolysis with hydroxylamine were freeze-dried and subjected to a second hydrolysis step with an excess of CNBr in the presence of 0.1 M-HCl at room temperature in the dark. In a typical experiment 2.5 mg of the hydroxylamine-cleaved protein was treated with 1 ml of CNBr solution (see above for experimental details). Portions were removed at the indicated intervals, subjected to SDS/PAGE and assayed for activity.

SDS/PAGE

Peptide fragments were separated by gradient SDS/PAGE as described by Fling & Gregerson (1986) with a high-molarity (3 M-)Tris/HCl buffer at pH 8.8. The resolving gel contained 8–25 % acrylamide and the stacking gel 5 % acrylamide. Protein or peptide samples were dissolved in sample buffer and boiled for 10 min before application. The running buffer consisted of 50 mM-Tris/192 mM-glycine containing 0.1 % SDS. The samples were dissolved in sample buffer [0.2 M-Tris/HCl buffer, pH 6.8, containing 3 % (w/v) SDS, 30 % (v/v) glycerol, 6 % (v/v) 2-mercaptoethanol and 0.01 % Bromophenol Blue]. Electrophoresis was carried out for 18 h at a constant current of 12 mA.

Staining and blotting

After electrophoresis, gels were removed from the mould, and portions were either stained with Coomassie Brilliant Blue R or by silver staining (Merril *et al.*, 1981) or subjected to blot transfer.

Blotting on to nitrocellulose membrane filters was carried out in a gradient electric field with transfer buffer, which contained 16 mM-Tris and 0.12 M-glycine without SDS, as previously described (Gershoni & Palade, 1983; Gershoni *et al.*, 1985). Blots were stained with 0.1 % Amido Black, examined for carbohydrate content or tested for biotin-binding activity as described below.

The presence of the mannose-containing carbohydrate chain of avidin was carried out by the two-step concanavalin A–peroxidase method of Clegg (1982).

Electroblotting on to PVDF membranes for sequencing was carried out by the method of Matsudaira (1987). After electrophoresis the gel was soaked for 5 min in the transfer buffer, which contained 10 mM-Caps and 10 % (v/v) methanol at pH 11. The membranes were rinsed with methanol for 3 min and stored in the transfer buffer. Electroblotting was carried out for 4 h at 300 mA at 4 °C. The membrane was then washed in water for 5 min, stained with 0.1 % Coomassie Blue in 50 % (v/v) methanol for 5 min, and destained in 50 % (v/v) methanol/10 % (v/v) acetic acid. Finally, the membrane was washed in water and air-dried before sequencing.

Biotin-binding assays

In order to determine the biotin-binding capacity of a given sample, two different assay systems were developed. In one, the relative biotin-binding capacities of individual SDS/PAGE-separated bands were determined by initial interaction with biotinylated BSA (B-BSA) followed by radiolabelled streptavidin. In the second approach, a competitive binding assay was developed in order to determine the biotin-binding capacity of the synthetic peptides. The respective assays were selected for a given purpose on the basis of either convenience or suitability of the assay conditions. All stages described here were performed at room temperature with shaking.

Biotin-binding activity on blots. In order to determine the biotin-binding capacity of protein fragments, an ultrasensitive biotin-binding assay was developed. Strips or sheets of nitrocellulose membrane that contained the blotted protein fragments (after SDS/PAGE) were quenched for 1 h in 0.1 % heat-treated milk in phosphate-buffered saline (0.15 M-NaCl/10 mM-sodium phosphate buffer, pH 7.4). The nitrocellulose strips were rinsed with phosphate-buffered saline and incubated for 1 h with B-BSA (3–5 μg/ml) in 0.1 % milk in phosphate-buffered saline. The blots were washed for 1 h with phosphate-buffered saline (with five to seven buffer changes) and incubated with radioiodinated streptavidin (500000 c.p.m./ml) in phosphate-buffered saline. The nitrocellulose strips were washed extensively for 1 h and air-dried. The blots were then exposed to X-ray film for 24 h at −70 °C with an intensifying screen. The signal associated with avidin monomers usually appeared within a few hours, whereas that of the fragments generally appeared the following day and became stronger within a few days. The specificity of the interaction was checked by adding an excess of unlabelled biotin to the B-BSA solution.

Competitive biotin-binding assay of synthetic peptides. The biotin-binding capacity of the synthetic peptides was determined with a competitive-type assay system that involved immobilization of B-BSA on nitrocellulose membrane filters or on flexible micro-titre plates, followed by incubation with iodinated streptavidin in the presence of the desired synthetic peptide. A weighed sample of the synthetic peptide was initially dissolved in small amounts of either 0.1 M-HCl or 0.1 M-NaOH; the former was then diluted with phosphate-buffered saline and the latter with double-distilled water.

A solution of B-BSA (1 mg/ml) was distributed in 5 μl portions on to nitrocellulose filters (2 cm × 2 cm) and air-dried. The blots were then quenched for 1–2 h in 0.1 % milk in phosphate-buffered saline, washed with phosphate-buffered saline and then incubated with radioiodinated streptavidin (500000 c.p.m./ml) containing various concentrations of one of the synthetic peptides. Parallel controls were run with radioiodinated streptavidin either alone (positive control) or combined with biotin (blank). Negative controls consisted of peptides derived from proteins that do not bind biotin. The incubation was carried out at room temperature for 1–2 h. The filters were washed extensively for 1 h (five changes), air-dried and their radioactivities counted as above with a γ-radiation counter.

For micro-titre plates, the assay was carried out as above with some modifications. Portions (5 μg) of B-BSA were immobilized in the wells (16–20 h incubation). Wash and sample solutions consisted of 0.2 ml. Wells were washed extensively with phosphate-buffered saline and dried carefully. Each well was cut out and its radioactivity counted as above with a γ-radiation counter.

N-Terminal sequencing

In solution. The *N*-terminal sequence of a given protein sample in solution (about 100 pmol) was determined by Edman degradation (three to five cycles per sample) in an Applied Biosystems model 470A protein peptide sequencer.

On PVDF membranes. After electroblotting of the SDS/PAGE-separated fragments on to PVDF membranes (see above), stained bands were excised, and the contents were subjected to

sequencing. Before Edman degradation, the Sequenator filter was washed with a solution (30 μl) containing Polybrene (10%) and NaCl (0.67%).

C-Terminal analysis

Carboxypeptidase Y treatment. The *C*-terminus was identified with the use of carboxypeptidase Y (1:50 ratio) as described by Hayashi (1977), modified as follows. Samples of protein (1 mg) were dissolved in 1 ml of 0.1 M-pyridine/acetate buffer, pH 5.5. The protein samples were first incubated at 100 °C for 10 min, and then, after cooling, they were digested at 37 °C with carboxypeptidase Y (20 μg in water or 10 mM-sodium phosphate buffer, pH 7.0). Portions (0.2 ml) were withdrawn at zero time and at selected intervals and mixed with 1 ml of 10% (w/v) trichloroacetic acid to stop the reaction. The precipitates were then removed by centrifugation, and the supernatant fluids were extracted with diethyl ether until the pH of the solution of each sample was neutral. The samples were dried, dissolved subsequently in 0.1 ml of 0.2 M-sodium citrate buffer, pH 2.2, and amino acid analysis was performed as detailed below.

Digestion with carboxypeptidases A and B. Digestion with carboxypeptidases A and B was performed as described by Ambler (1967). Samples of protein in the appropriate buffer solution (1 mg/ml) were incubated at 100 °C for 10 min, cooled and digested at 37 °C with the desired enzyme (1:50 ratio). Carboxypeptidase A treatment was performed in 0.2 M-ammonium bicarbonate buffer, pH 8.0, and digestion with carboxypeptidase B was carried out in 0.2 M-*N*-ethylmorpholine/acetate buffer, pH 8.5.

Hydrazinolysis. The *C*-terminal amino acid was confirmed by hydrazinolysis (Fraenkel-Conrat & Tsung, 1967). Dried protein samples (1 mg) were hydrolysed with an excess of hydrazine (200 μl treated with N_2 gas) at 110 °C for 5 h in vacuum-sealed Pyrex tubes and dried. Samples were then dissolved in 0.1 ml of 0.2 M-sodium citrate buffer, pH 2.2, and amino acid analysis was performed.

Miscellaneous methods

Proteins were biotinylated with the use of biotin *N*-hydroxysuccinimide ester, according to published procedures (Bayer & Wilchek, 1990*b*).

Iodination was carried out by the chloramine-T method with 1 mCi of $Na^{125}I$/100 μg of the desired protein, according to the procedure of Hunter & Greenwood (1962).

Peptides were synthesized by the Merrifield (1964) method in a solid-phase 430A peptide synthesizer (Applied Biosystems). The peptides were synthesized with the use of the appropriate *N*-t-butyloxycarbonyl-amino acid phenylacetamidomethyl resin esters as the immobilized matrix. The preparations were characterized by SDS/PAGE, h.p.l.c. and amino acid analysis.

Amino acid analyses were performed on a Dionex D-500 amino acid analyser (Durram Instrument Corp., Palo Alto, CA, U.S.A.). Protein samples were hydrolysed with 6 M-HCl at 110 °C for 22 h in vacuum-sealed Pyrex tubes. Tryptophan residues were determined after hydrolysis in 4 M-methanesulphonic acid under vacuum at 110 °C for 22 h. Cysteine residues were detected as cysteic acid after hydrolysis with dimethyl sulphoxide/conc. HCl (1:1, v/v) under vacuum at 110 °C for 22 h.

Protein concentration was determined by either the Lowry *et al.* (1951) or the Bradford (1976) procedure.

RESULTS

In order to produce defined fragments of the avidin, we first employed enzymic and then chemical methods in attempts to decrease the size of the subunit. Several well-established proteolytic enzymes were employed for this purpose. None, however, was capable of cleaving either avidin or streptavidin into reliably discrete fragments that retained biotin-binding activity. We therefore decided to try chemical approaches, which proved much more effective to this end. Specifically, hydroxylamine and CNBr cleavage of avidin (either alone or in succession) resulted in the production of a series of defined fragments, the identity of which was determined by protein-sequencing techniques. Some of these fragments were shown to bind biotin selectively. The functional region of biotin binding was further resolved by synthesizing selected peptide segments of avidin and streptavidin. The interaction of the synthetic peptides with biotin was determined by a competitive binding assay.

Development of an ultrasensitive biotin-binding assay on blots

We assumed that at each chemical or enzymic degradation step of avidin the affinity of biotin to the resultant fragments of avidin may decrease drastically. The establishment of a very sensitive efficient probe for determining the biotin-binding capacity of protein fragments was therefore essential. Initially, we tried an enzyme-based technique using the interaction of blotted fragments with biotinyl-(alkaline phosphatase) (Hiller *et al.*, 1987), but it soon became clear that this approach was too insensitive to determining the biotin-binding capacity of avidin fragments. The use of [*carbonyl*-^{14}C]biotin to label blotted samples directly also proved to be of relatively low sensitivity. We therefore developed an iodinated form of biotin, biotinyl[^{125}I-iodo]histamine (Groman *et al.*, 1990), which was indeed sufficiently sensitive as a probe, but in our hands resulted in relatively high levels of non-specific binding and high background.

More recently we were able to develop a new blot-overlay technique, based on sequential treatment with B-BSA and ^{125}I-streptavidin. This approach proved to be extremely sensitive and specific with little or no background (Fig. 1). We tried to improve this method further by developing a more direct procedure based on radiolabelled biotinylated proteins (i.e. biotinylated ^{125}I-BSA or biotinylated ^{125}I-immunoglobulin). The idea behind this approach was that direct incubation with the iodinated probe

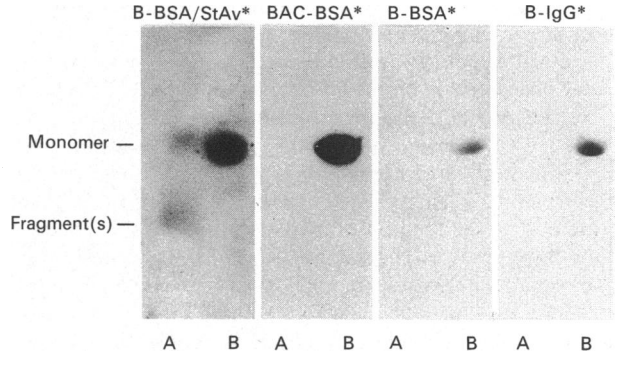

Fig. 1. Activity measurements of avidin and avidin fragments made with various iodinated biotinylated probes

Native avidin (30 μg; lanes B) and avidin that had been treated with hydroxylamine for 6 days (150 μg; lanes A) were resolved by SDS/8–25%-PAGE, blotted on to nitrocellulose membrane filters and assayed for biotin-binding activity with the indicated biotinylated probes, i.e. two-step incubation with B-BSA (3 μg/ml) followed by iodinated streptavidin (B-BSA/StAv*), direct incubation with the long-chained biotinyl amidohexanoyl derivative of ^{125}I-BSA (BAC-BSA*) and direct incubation with either biotinylated ^{125}I-BSA (B-BSA*) or biotinylated ^{125}I-immunoglobulin (B-IgG*). The iodinated proteins were used at 500000 c.p.m./ml.

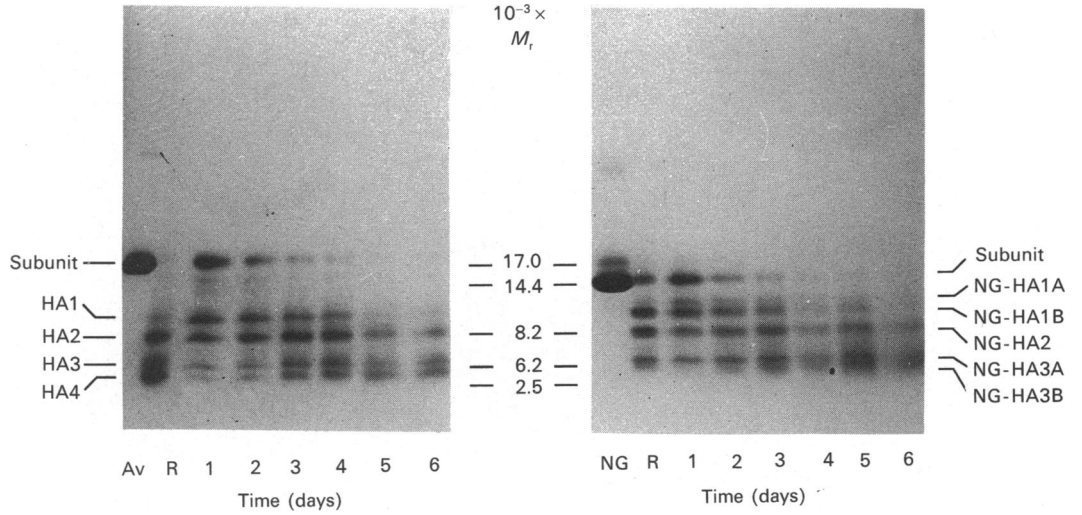

Fig. 2. Electrophoretic pattern of native and non-glycosylated avidin treated with hydroxylamine as a function of time

Portions of the reaction mixture (15 μg/lane) were examined at desired intervals (1 to 6 days as indicated), and were resolved by SDS/8–25%-PAGE. Avidin (Av), non-glycosylated avidin (NG) and previously prepared standards (R; incubated for 6 days with hydroxylamine) were included as controls. The markers used in this experiment to determine the M_r values presented in the figure consisted of myoglobin and its fragments as designated in the Materials and methods section.

would increase the sensitivity or intensity of the binding. A radiolabelled protein with attached biotin moieties containing extended spacers (specifically, biotinylated amidohexanoyl-BSA) was also tried and was found to interact much more effectively with the intact avidin subunit, resulting in higher signal levels than those generated by short-chain analogues. Nevertheless, all of these direct procedures failed to yield detectable levels of label for the avidin fragments. This may be explained by the small amounts of the iodinated probe used in the direct procedure; whereas in the sequential procedure we could use high amounts of the unlabelled probe (i.e. 3–5 μg of B-BSA/ml), the direct approach was limited by the effective concentration of the iodinated probes (maintained at 0.25–0.5 μg/ml to eliminate very high background levels). It thus appears that relatively high concentrations of the biotinylated probe are imperative for detection of the biotin-binding fragments in avidin, indicative of their highly diminished affinity for biotin.

Optimization experiments showed that the binding by avidin fragments reached a maximal level at a B-BSA concentration of 30 μg/ml, but with very high background levels. Optimal results (signal versus background) were achieved with 3–5 μg of B-BSA/ml.

The binding of B-BSA to avidin fragments on blots appears to be specific, since excess biotin prevented the binding. In addition, other polypeptides (which fail to recognize biotin) were not labelled by the sequential B-BSA-^{125}I-streptavidin treatment. Treatment with unmodified BSA failed to label avidin or avidin fragments.

Fragments obtained by hydroxylamine treatment

Treatment of proteins with hydroxylamine is known to cleave the Asn–Gly peptide bond (Bornstein, 1970); one such sequence is located at position 88–89 in avidin. It was therefore of interest to determine whether this approach could successfully sever avidin in a selective manner that would result in active biotin-binding peptides. With this procedure, only two fragments would be expected, i.e. peptides 1–88 and 89–128 (and probably the uncleaved subunit, i.e. residues 1–128, as a third SDS/PAGE band). However, when glycosylated avidin was treated with hydroxylamine at pH 9 at 45 °C, other bands were also visible on SDS/PAGE (Fig. 2). These were designated HA1, HA2, HA3 and HA4. This indicates that hydroxylamine treatment cleaves other peptide bonds in addition to (or instead of) the Asn–Gly position. By using the ultrasensitive biotin-binding assay, it was found that the fragment(s) that migrates in the vicinity of the HA2 band (8 kDa) was capable of specifically binding biotin. Concanavalin A staining on blots revealed that the HA2 fraction contains carbohydrate moieties as well (Fig. 3). Furthermore, it was found that fragments obtained after several days of incubation were capable of binding biotin at higher signal intensities than those obtained within 24 h of hydrolysis (results not shown), apparently owing to the accumulation of the low-M_r cleavage product(s). We therefore decided to focus our attention on the fragments obtained after several days of incubation. It is reiterated here that we were searching for fragment(s) of minimal size.

Various unsuccessful attempts (e.g. the use of biotin–Sepharose column chromatography, concanavalin A–Sepharose column chromatography, gel filtration on a Sephadex column as well as h.p.l.c.) were conducted to isolate relatively large amounts of these biotin-binding fragments. We therefore proceeded to identify and characterize the SDS-separated active and non-active fragments on PVDF membranes. The general purpose of this part of the research was to identify the major sites of hydroxylamine-induced cleavage and to categorize the resultant SDS/PAGE bands with respect to the corresponding peptide fragments produced, the salient question being which fragments actively bind biotin and which do not.

As mentioned above, SDS/PAGE of the reaction mixture (hydroxylamine-treated glycosylated avidin) revealed only four major fragments (plus the uncleaved subunit). Only upon PVDF staining was it found that some of the bands actually comprised subordinate bands. A lower-M_r band, termed HA5, was also detected as a very faint signal only upon Coomassie Brilliant Blue staining of PVDF membranes. A schematic representation of the Coomassie Brilliant Blue-stained hydroxylamine-treated avidin and non-glycosylated avidin preparations is presented in Fig. 4. For identification purposes, the stained PVDF-immobilized bands were sliced into fractions as illustrated in the Figure.

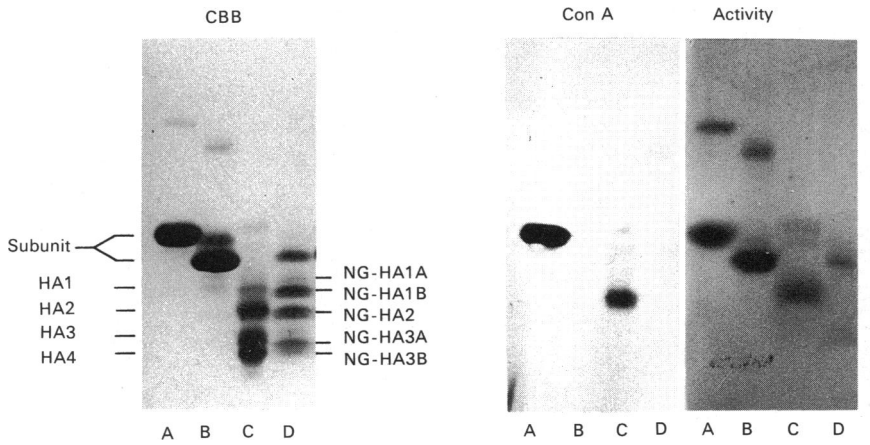

Fig. 3. Cleavage of native and non-glycosylated avidin with hydroxylamine

Portions from the dialysed reaction mixtures of native and non-glycosylated avidin (lanes C and D respectively) after 6 days of treatment with hydroxylamine were resolved by SDS/8–25%-PAGE. Native avidin and non-glycosylated avidin were included in lanes A and B respectively as controls. The gels were then either stained with Coomassie Brilliant Blue (CBB) or blotted on to nitrocellulose membrane filters. Blots were then either probed with the concanavalin A–peroxidase system for the presence of mannose-containing oligosaccharides (Con A) or probed with B-BSA followed by ^{125}I-streptavidin (Activity). For CBB and Con A treatments, 15 μg of each protein sample was used; for activity measurements, 30 μg samples were applied to lanes A and B, and 150 μg samples were applied to lanes C and D.

N-Terminal analysis revealed that the HA1A fraction contains two fragments, residues 58–128 and 1–57, and the HA1B fraction contains residues 43–104. The HA2A band consists mainly of a single peptide fragment (residues 1–42), but the HA2B band comprises two subcomponents, which correspond to residues 58–104 and residues 1–42 (overlapping material from the HA2A band). Further *N*-terminal analysis revealed that the HA3 band contained two fragments (residues 43–88 and 58–104, the latter representing overlapping material from the HA2B fraction). It was found that the bands HA4A and HA4B denote residues 58–88 and 105–128, and that the HA4C band includes the same two overlapping fragments plus a third unique fragment (residues 62–88). The lower-M_r band (designated HA5) contained two fragments, i.e. minor amounts of residues 89–104 and 1–12. The results of the identification of hydroxylamine-induced fragmentation of avidin are summarized in Table 1.

These results indicate that the chemical cleavage also occurred at other peptide bonds, as indicated in Table 2. It is also noteworthy that the Asn–Glu and Asp–Lys peptide bonds in avidin were very susceptible to hydroxylamine cleavage. In order to confirm the mild cleavage of the Asn–Glu peptide bond in avidin, we checked the action of hydroxylamine on other proteins that contained this sequence. For example, trypsinogen contains a single Asn–Glu peptide bond at position 67–68, which was found to be extensively cleaved within a 5 min incubation period. This again suggests the relative stability of the avidin molecule, since much longer exposure times to hydroxylamine were needed for efficient cleavage of the avidin Asn–Glu peptide bond. Unexpectedly, both Asn–Glu and Asp–Lys bonds were cleaved much more efficiently than the Asn–Gly peptide bond of avidin. Additional cleavage sites were at the Asn–Asp peptide bond and, perhaps surprisingly, at the Gln–Pro peptide bond (albeit to small extents). This indicates a relatively broad specificity of hydroxylamine cleavage, despite previously reported claims to the contrary.

In parallel with these experiments, non-glycosylated avidin was also subjected to hydroxylamine treatment. It was hoped that the absence of the carbohydrate groups from the *N*-terminal peptide fragments would aid in the identification of the relevant active biotin-binding region(s) of the avidin molecule. In this case, too, several days of incubation were needed in order to obtain defined low-M_r active fragments (Fig. 2). Five major bands (plus the uncleaved subunit) were obtained. These were termed NG-HA1A, NG-HA1B, NG-HA2, NG-HA3A and NG-HA3B. By using the ultrasensitive biotin-binding assay it was found that (in addition to the uncleaved non-glycosylated subunit) the lower-M_r bands [designated NG-HA3A and/or NG-HA3B (M_r approx. 4000)] were capable of binding biotin (Fig. 3, lanes D). *N*-Terminal analysis on PVDF membranes revealed that the NG-HA3A band comprises residues 1–42 and 43–88. the NG-HA3B band contains a complex mixture of fragments, namely residues 58–88, 89–128 and 105–128 (Table 1), as well as overlapping portions of the fragment representing residues 1–42. Further *N*-terminal analysis revealed that the NG-HA1A band contains residues 1–88 and 58–128; the NG-HA1B band contains residues 43–104, and the NG-HA2 band contains residues 1–57 and 58–104. The non-glycosylated equivalent to the HA4C and HA5 bands (which we assumed would contain residues 1–12, 62–88 and 89–104) could not be detected by either SDS/PAGE staining and/or PVDF staining (Fig. 4). This could reflect the fact that the hydrolysis experiments performed on glycosylated and non-glycosylated avidin were carried out separately and the conditions may not have been precisely equivalent. Nevertheless the results suggest that hydroxylamine cleaves non-glycosylated avidin in a manner very similar to that of the fully glycosylated protein (with the possible exception of the Gln–Pro peptide bond).

Fragments obtained by CNBr treatment

In addition to the above-described results for hydroxylamine cleavage of avidin, CNBr cleavage of the protein led to the accumulation of complementary evidence that served to define further the region of the avidin molecule in which low levels of biotin-binding activity are maintained after fragmentation. CNBr cleavage of both native (glycosylated) avidin and the non-glycosylated protein was performed, and the respective fragments obtained are presented schematically in Fig. 5.

Native avidin was treated with CNBr in the presence of formic acid (70%) for 24 h at room temperature (Gross & Witkop, 1961). SDS/PAGE of the reaction mixture in the absence of 2-mercaptoethanol (Fig. 6*a*, lane A) revealed the presence of three bands (Table 3, part *a*). However, in the presence of 2-mercaptoethanol, SDS/PAGE of the reaction mixture revealed

Table 1. Fragments resulting from hydroxylamine treatment of native and non-glycosylated avidin

The hydroxylamine-treated material was separated by gradient SDS/PAGE, blotted on to PVDF membranes and stained with Coomassie Brilliant Blue, and the resultant bands (as shown schematically in Fig. 4) were excised and subjected to *N*-terminal sequencing. The data show only the peptide fragments unique to a given band; overlapping fragments that appear in other bands are not presented.

Band	$M_{r(obs.)}$*	Proposed fragment (residues)	$M_{r(calc.)}$†
Native avidin			
Uncleaved subunit	18000	1–128 (+sugar)	15300
HA1A	11000	58–128	8300
		1–57 (+sugar)	7500
HA1B	10000	43–104	7100
HA2A	8500	1–42 (+sugar)	6300
HA2B	8000	58–104	5500
HA3	7000	43–88	5300
HA4A, HA4B	5000	58–88	3600
		105–128	2800
HA4C	4500	62–88	3100
HA5	2000	89–104	1900
		1–12	1400
Non-glycosylated avidin			
Uncleaved subunit	15000	1–128	13800
NG-HA1A	11000	1–88	9700
		58–128	8300
NG-HA1B	10000	43–104	7100
NG-HA2	8000	1–57	6000
		58–104	5500
NG-HA3A	7000	43–88	5300
		1–42	4800
NG-HA3B	5000	89–128	4700
		58–88	3600
		105–128	2800

* Based on the electrophoretic migration on SDS/PAGE, relative to markers of known M_r (see Fig. 2).

† Based on the sum of the amino acid residues that comprise each peptide and on an estimated M_r of 1500 for the carbohydrate moiety where applicable (based on three *N*-acetylglucosamine and five mannose residues per oligosaccharide).

Table 2. Hydroxylamine-cleavage sites in avidin

Bond cleaved	Position	Cleavage efficiency
Asn–Asp	12–13	Minor
Asn–Glu	42–43	Major
Asp–Lys	57–58	Major
Gln–Pro	61–62	Minor
Asn–Gly	88–89	Minor
Asn–Asp	104–105	Moderate

five bands (Fig. 6*b*, lane A) designated CN1a, CN2a, CN1b, CN2b and CN3, which were deduced to represent residues 1–128, 1–96, 19–128, 19–96 and 97–128 respectively (Table 3, part *b*). The free glycopeptide (residues 1–18) was found to co-migrate with the CN2b band (residues 19–96), as revealed by concanavalin

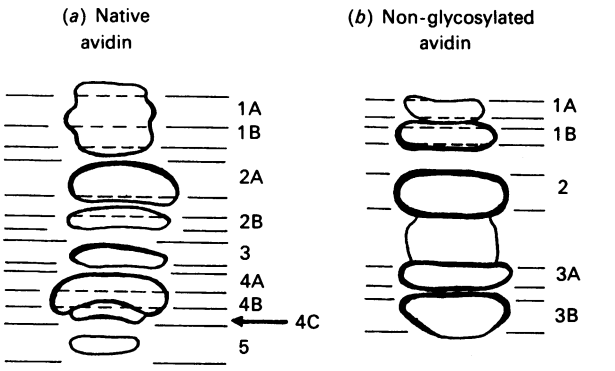

Fig. 4. Schematic representation of Coomassie Brilliant Blue-stained hydroxylamine-cleaved fragments of (*a*) native avidin and (*b*) non-glycosylated avidin on PVDF

The scheme served as a guide for the identification of the fragments mentioned in Table 1.

A staining on blots (Fig. 7). The relative migratory pattern of the small glycopeptide is much slower than would be expected from its actual molecular size.

Chromatography of the CNBr-cleaved preparation on Sephadex G-75 under non-reducing conditions gave two distinct peaks (Fig. 8). SDS/PAGE (in the presence of 2-mercaptoethanol) showed that the second peak contains only the 4000-M_r *C*-terminal peptide (CN3; Fig. 6, lanes 56–67). The first peak represents a partially separated mixture of all the other peptides: bands CN1a and CN1b appeared to be co-eluted and were enriched in the initial fractions of peak I, whereas bands CN2a and CN2b (which also were co-eluted) were enriched in the latter fractions of the peak (Fig. 6, lanes 33–53).

By using the ultrasensitive iodination assay combined with nitrocellulose blot transfer, it was found that fragments CN1a, CN1b, CN2a, CN2b and CN3 did not bind biotin extensively (Fig. 7, lanes A). It was not clear, however, whether the lack of biotin binding in these fragments was a consequence of a simple reduction in size of the protein molecule to form inactive components or whether these observations reflect a chemical alteration of binding-site residues caused by the conditions employed for digestion, e.g. the use of 70% formic acid. In this context, formic acid may act as an oxidizing reagent, and a critical amino acid (e.g. tryptophan) may be modified, thus affecting the biotin-binding capacity of the fragment(s). Essentially identical results were obtained for CNBr/formic acid treatment of non-glycosylated avidin, which indicate that the sugar moieties do not affect the pattern of cleavage (results not shown).

In the light of the above results, CNBr cleavage was performed again on avidin and non-glycosylated avidin, but this time under milder conditions, i.e. in the presence of 0.1 M-HCl rather than formic acid (Spande *et al.*, 1970). Indeed, by using the ultrasensitive iodination assay combined with nitrocellulose blot transfer, it was found that, in this case, the bands representing residues 1–96, 19–96, 19–128 and 1–128 (the intact non-glycosylated protein) were all capable of specifically binding biotin, whereas the 4000-M_r *C*-terminal fragment (residues 97–128) failed to bind biotin (Fig 7, lanes B–E). Activity profiles suggested that the small *N*-terminal CNBr-cleavage fragment (residues 1–18) is also inactive, since the activity patterns of both the glycosylated and the non-glycosylated material appear to be very similar (compare lanes D and E); had this fragment shown a high degree of activity, the SDS/PAGE mobility pattern of the two preparations would be expected to be very different, and this

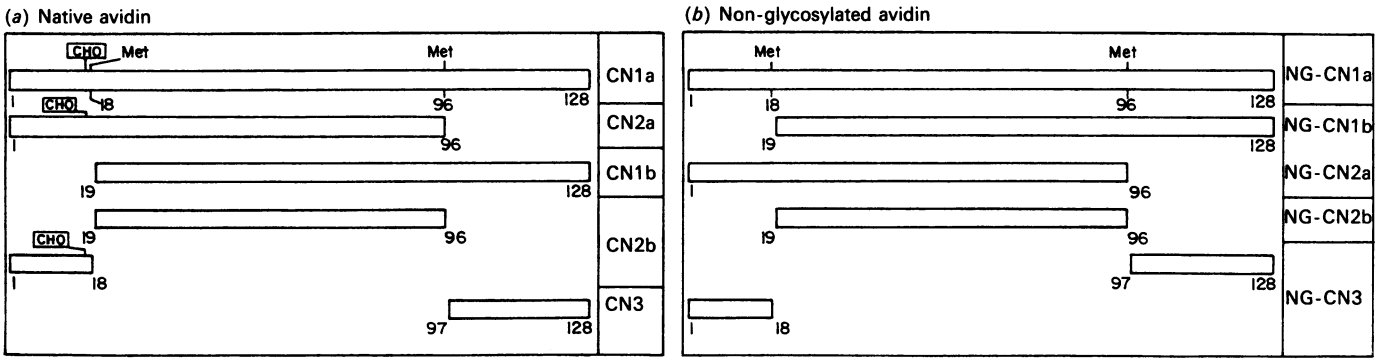

Fig. 5. Schematic description of fragments obtained from CNBr cleavage of (*a*) native avidin and (*b*) non-glycosylated avidin

The scheme shows the relative positions of the carbohydrate moiety (CHO) and the two methionine residues in avidin.

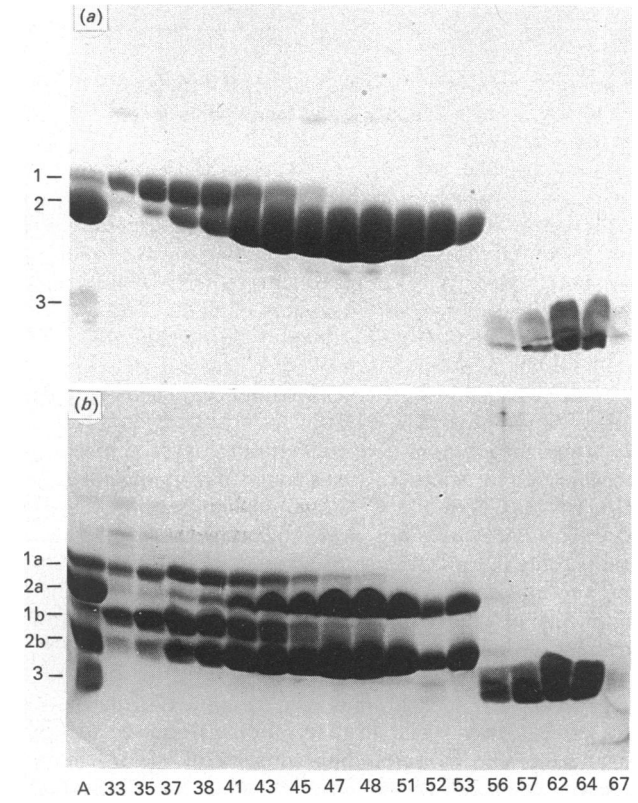

Fig. 6. Electrophoretic mobility of CNBr-cleaved fractions of native avidin after chromatography on Sephadex G-75

The CNBr-treated reaction mixture (lane A) was subjected to gel filtration (see Fig. 8) as described in the text. SDS/15%-PAGE was carried out on the indicated fractions (20 μg of protein/lane) either in the absence (panel *a*) or in the presence (panel *b*) of 2-mercaptoethanol. The gels were then stained with Coomassie Brilliant Blue.

Table 3. SDS/PAGE of CNBr-cleavage fragments of native avidin

Portions from the CNBr (formic acid) reaction mixture with avidin were subjected to SDS/PAGE after incubation at 100 °C for 10 min either in the absence or in the presence of 2-mercaptoethanol as indicated. The gels were stained with Coomassie Brilliant Blue. The identity of given bands is based on the published sequence and on the assumed cleavage sites, confirmed later by sequencing.

Band	M_r*	Fragment (residues)
(*a*) Without 2-mercaptoethanol		
CN1	18000	1–128+[1–18 and 19–128 (disulphide-linked)]
CN2	15000	1–96+[1–18 and 19–96 (disulphide-linked)]
CN3	4000	97–128
(*b*) With 2-mercaptoethanol		
CN1a	18000	1–128 (intact subunit)
CN2a	15000	1–96
CN1b	12000	19–128
CN2b	8000	19–96 1–18
CN3	4000	97–128

* Based on the electrophoretic migration on SDS/PAGE, relative to markers of known M_r.

difference should have been detected by the ultrasensitive binding assay. The possibility also remains that the peptide is not amenable to blotting, owing to its very small size.

In comparing HCl with formic acid as a medium, the pattern of CNBr cleavage was found to be rather different (Fig. 7 and Table 4). On the basis of the corresponding SDS/PAGE profiles, it was estimated that in the presence of formic acid the extent of cleavage at Met-18 and Met-96 was about 35% and 60% respectively, resulting in a high content of the 1–96, 19–96 and 97–128 residues. In contrast, cleavage in the presence of 0.1 M-HCl occurred mainly at Met-18 (48% versus 13% at Met-96), resulting in a relatively high content of the fragment that contains residues 19–128 (Table 4). Longer incubation periods failed to effect more extensive levels of cleavage (see Fig. 7). Essentially the same pattern was obtained for non-glycosylated avidin, indicating further that the sugar residues do not markedly affect the character of the observed cleavage.

Owing to the increased biotin-binding activity observed in the fragments produced by CNBr treatment in the presence of HCl, we concentrated on the isolation and characterization of the active fragments obtained with this system. Attempts to isolate these bands by gel filtration employing a buffer system that contains 2-mercaptoethanol and SDS (0.1% each) were not successful. We therefore characterized the CNBr-cleavaged fragments of glycosylated and non-glycosylated avidin on PVDF in a manner similar to that described above for hydroxylamine-cleaved fragments. *N*-Terminal analysis of the SDS/PAGE separated fragments confirmed the assignments of the respective bands as listed in Table 4.

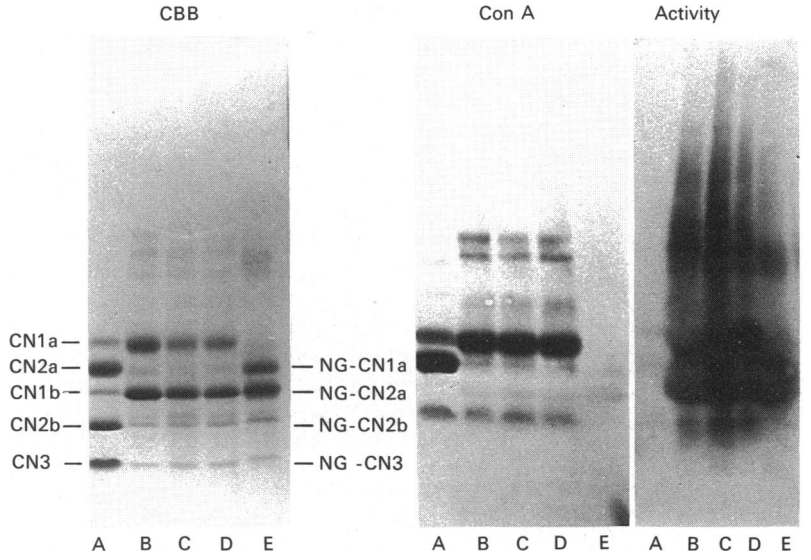

Fig. 7. Sugar content and biotin-binding activity of CNBr-cleaved fragments derived from native and non-glycosylated avidin

Portions from reaction mixture, obtained after incubation in the presence of formic acid for 1 day (lanes A), in the presence of 0.1 M-HCl/acetonitrile for 1 day (lanes B) and 4 days (lanes C) and in the presence of 0.1 M-HCl without acetonitrile for 1 day (lanes D), were resolved by SDS/8–25% -PAGE. Non-glycosylated avidin treated with CNBr/0.1 M-HCl for 1 day was included in lanes E. The gels were then either stained with Coomassie Brilliant Blue (CBB) or blotted on to nitrocellulose membrane filters. Blots were then either probed sequentially with concanavalin A and peroxidase and examined for peroxidase activity (Con A) or probed with B-BSA followed by ^{125}I-streptavidin (Activity). For the Coomassie Brilliant Blue and concanavalin treatments 15 μg of each protein/lane was used, whereas for activity measurements, 150 μg/lane was used. The NG-CN1b fragment (not shown in the Figure), which is equivalent to the CN1b fragment, co-migrates with the NG-CN2a fragment.

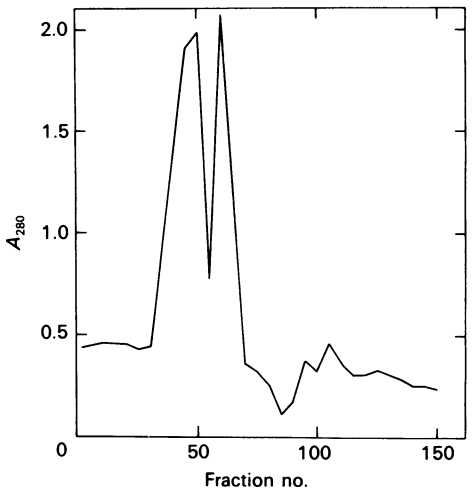

Fig. 8. Chromatographic pattern of CNBr-treated native avidin applied to a Sephadex G-75 column

Elution was accomplished with 30% acetic acid. Fractions of size 1.3 ml/tube were collected.

Table 4. Efficiency of fragment production by CNBr treatment of native and non-glycosylated avidin

The designated CNBr-treated preparation (in either formic acid or HCl) was subjected to SDS/PAGE and stained with Coomassie Brilliant Blue. The relative amount of material in each band was estimated by densitometry and expressed as a percentage of total absorbance. The band representing residues 1–18 was not visible (N.V.), and was not included in the calculations.

Band	M_r*	Fragment (residues)	Relative amount (%) Formic acid	HCl
Native avidin				
CN1a	18000	1–128	5	40
CN2a	15000	1–96	30	5
CN1b	12000	19–128	5	40
CN2b	8000	19–96	30	7.5
		1–18	N.V.	N.V.
CN3	4000	97–128	30	7.5
Non-glycosylated avidin				
NG-CN1a	15000	1–128	5	40
NG-CN1b	12000	19–128	5	40
NG-CN2a	12000	1–96	30	5
NG-CN2b	8000	19–96	30	7.5
NG-CN3	4000	97–128	30	7.5
		1–18	N.V.	N.V.

* Based on the electrophoretic migration on SDS/PAGE, relative to markers of known M_r.

Fragments obtained by sequential degradation with CNBr and hydroxylamine

Our search for the smallest possible active fragment(s) of avidin prompted further cleavage of the active fragments obtained by CNBr treatment with hydroxylamine. For this purpose, CNBr-cleaved fragments of glycosylated and non-glycosylated avidin (cleavage performed in the presence of 0.1 M-HCl) were subjected to hydrolysis with hydroxylamine at 45 °C for 5 days (Fig. 9).

Comparison of the pattern of cleavage and the activity profiles of these two types of avidin assisted in the identification of glycosylated fragments. This is particularly important in this case, where the additional cleavage sites would theoretically lead to a large number of fragments.

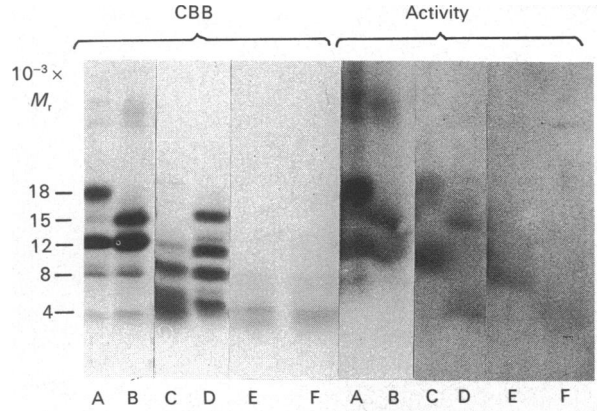

Fig. 9. Sequential CNBr–hydroxylamine treatment of native and non-glycosylated avidin

Portions from the reaction mixtures of native avidin (lanes A, C and E) and non-glycosylated avidin (lanes B, D and F) were subjected to the following treatments: CNBr in the presence of 0.1 M-HCl (lanes A and B); hydroxylamine for 6 days (lanes C and D); sequential degradation with both modes of cleavage (lanes E and F). The samples were resolved by SDS/8–25%-PAGE. The gels were then either stained with Coomassie Brilliant Blue (CBB) or blotted on to nitrocellulose membrane filters (Activity). Blots were then probed with B-BSA followed by ^{125}I-streptavidin. For Coomassie Brilliant Blue staining 20 μg of protein/lane was used, whereas for activity measurements 150 μg/lane was used. The M_r values of some of the major fragments are shown for reference.

By using this approach, it was found that CNBr-cleaved fragments derived from native and non-glycosylated avidin were indeed further cleaved by hydroxylamine. In both cases it was found that the higher-M_r fragments were further cleaved to lower-M_r fragments (generally migrating to the 8000-M_r and 4000-M_r regions), as revealed by Coomassie Brilliant Blue staining (Fig. 9, lanes E and F). However, by using the ultra-sensitive biotin-binding assay, it was found that a fragment(s), positioned at the 8000-M_r region obtained by the cleavage of the glycosylated avidin, was capable of binding biotin, whereas in the non-glycosylated avidin a biotin-binding fragment(s) appeared in the 4000-M_r region of the gel. The complexity of the fragments obtained by the sequential procedure precluded their separation and identification by the methodology used in this study.

We also investigated whether alteration of the sequence of co-chemical cleavage would lead to a similar result. For this purpose glycosylated avidin and non-glycosylated derivatives, obtained by hydroxylamine cleavage after 6 days, were subjected to subsequent hydrolysis with an excess of CNBr in the presence of 0.1 M-HCl as a function of time. The SDS/PAGE pattern (staining and activity) of the reversed sequential procedure (hydroxylamine–CNBr treatment) appeared to be essentially identical with that of fragment(s) produced by sequential CNBr–hydroxylamine treatment (results not shown).

Fragmentation of bacterial streptavidin subunits

Native (unprocessed) streptavidin was sensitive to the action of a variety of proteinases (Bayer *et al.*, 1989), which converted the protein into truncated forms no smaller than the commercially available 'core' streptavidin. We tried to obtain smaller streptavidin fragments by using extended incubation periods, but further degradation of core streptavidin into defined low-M_r bands by either enzymic or chemical means proved to be unproductive in the presence of SDS. Extended treatment with proteinase K (5 days at 37 °C) resulted in extensive degradation of both the 18000-M_r and 14000-M_r forms of streptavidin. Defined bands, however, were not obtained; rather, a smear of proteinaceous material was observed within an M_r range of about 5000–7000. No biotin-binding activity could be detected. A similar picture emerged upon treatment of streptavidin with hydroxylamine, which yielded a smear of polypeptides roughly within an M_r range of 7000–8000. CNBr treatment of streptavidin was not conducted, since, as mentioned above, this protein lacks methionine residues. Therefore, until now, the smallest active fragment that we can consistently detect in streptavidin is the 14000-M_r subunit of the truncated molecule.

Biotin-binding activity of synthetic peptides

In order to confirm and/or to complement the studies obtained by hydroxylamine and CNBr cleavage of avidin, we synthesized selected peptides from avidin and streptavidin (Fig. 10). The synthetic sequences were designed to answer questions that were difficult to address by means of the cleavage studies. On the one hand, we chose sequences 1–19 and 26–38 of avidin in order to differentiate between the tyrosine- and carbohydrate-containing portions of the *N*-terminus. On the other hand, we were interested to see whether we could prepare a relatively large tryptophan-containing peptide from streptavidin, since we failed to prepare streptavidin fragments via chemical or enzymic means. The fragment (residues 77–124) that we chose to synthesize from streptavidin contained all the tryptophan residues of interest (according to X-ray or chemical-modification studies or from analysis of conserved residues) but lacked the tyrosine residue analogous to that in avidin.

The idea in this series of experiments was to test the biotin-

Streptavidin-(77–124)-peptide

Val-Ala-Trp-Lys-Asn-Asn-Tyr-Arg-Asn-Ala-His-Ser-Ala-Thr-Thr-Trp-Ser-Gly-Gln-Tyr-Val-Gly-Gly-Ala-Glu-Ala-Arg-Ile-Asn-Thr-Gln-Trp-Leu-Leu-Thr-Ser-Gly-Thr-Thr-Glu-Ala-Asn-Ala-Trp-Lys-Ser-Thr-Leu

Avidin-(1–19)-peptide

Ala-Arg-Lys-Cys-Ser-Leu-Thr-Gly-Lys-Trp-Thr-Asn-Asp-Leu-Gly-Ser-Asn-Met-Thr

Avidin-(26–38)-peptide

Arg-Gly-Glu-Phe-Thr-Gly-Thr-Tyr-Ile-Thr-Ala-Val-Thr

Fig. 10. Amino acid sequences of the synthetic peptides prepared in this work

Amino acid residues that are the same in both avidin and streptavidin are underlined. Avidin-(1–19)-peptide contains all of domain I (as described by Bayer & Wilchek, 1990*a*), and avidin-(26–38)-peptide contains most of domain II. The large 48-residue streptavidin-(77–124)-peptide contains all of domains IV and V and parts of domains III and VI. This peptide includes the four tryptophan residues known to be involved in the biotin-binding site of streptavidin (Weber *et al.*, 1989).

Control peptide A

Val-Glu-Val-Pro-Gly-Ser-Gln-His-Ile-Asp-Ser-Gln-Lys-Lys

Control peptide B

Trp-Lys-His-Trp-Val-Tyr-Tyr-Thr-Cys-Cys-Pro-Asp-Thr-Pro-Tyr-Leu-Asp

Fig. 11. Amino acid sequences of peptides A and B used as controls in the competitive binding assay

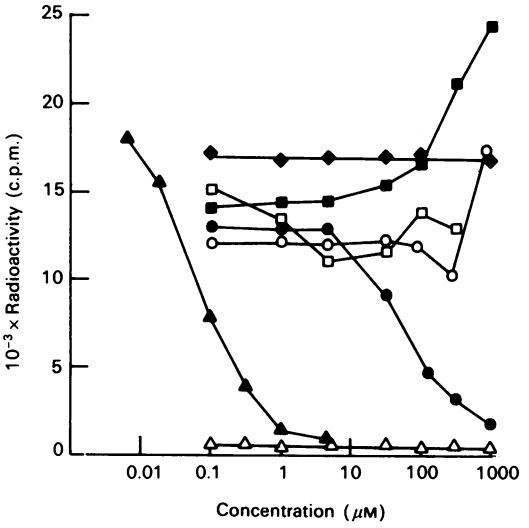

Fig. 12. Biotin-binding activity of the synthetic peptides

B-BSA was immobilized on flexible micro-titre plates and incubated with iodinated streptavidin in the presence of different concentrations of the indicated synthetic peptide: ●, avidin-(26–38)-peptide; ○, avidin-(1–19)-peptide; ◆, streptavidin-(77–124)-peptide; ■, control peptide A; □, control peptide B; ▲, streptavidin; △, biotin. The sequences of the peptides used in this experiment are shown in Figs. 10 and 11.

binding activity of each of these fragments. Since these peptides were generally quite small, their direct assay with B-BSA proved problematic; consequently, we developed a competitive binding assay in its stead. The assay was based on the interference afforded by a given synthetic peptide preparation on the binding between streptavidin and an immobilized form of B-BSA. For this purpose, B-BSA was immobilized on flexible microtitre plates (or on nitrocellulose membrane filters) and then incubated with a solution containing a mixture of iodinated streptavidin with different concentrations of one of the following sample materials: peptides 26–38 and 1–19 from avidin; peptide 77–124 from streptavidin; two other unrelated synthetic peptides (designated A and B) as negative controls (Fig. 11); unlabelled streptavidin as a positive control; biotin as a blank.

The results (Fig. 12) clearly showed that, among the synthetic peptides that we prepared, only peptide 26–38 from avidin was capable of binding biotin. The other synthetic peptides (1–19 and 77–124) appeared not to be capable of binding biotin from the results of the competition assay. Control peptides A and B failed to bind biotin, as expected. It is noteworthy that in the case of control peptide A and synthetic peptide 1–19 very high concentrations led to elevated signal levels, which may suggest non-specific aggregation and adsorption of the radiolabelled streptavidin, which would then bind in oligomeric form to the B-BSA-coated plates. Competition with unlabelled streptavidin dramatically decreased the levels of biotin binding by the iodinated form. The results presented in Fig. 12 indicate that when unlabelled streptavidin was added only micromolar concentrations were needed in order to abolish the biotin binding, whereas in the case of the synthetic peptide 26–38 millimolar concentrations were required. This appears to reflect the relatively low biotin-binding affinity exhibited by this synthetic peptide.

DISCUSSION

The purpose of this study was to examine whether fragments of avidin and streptavidin can be prepared that still bind biotin. According to X-ray-crystallographic studies (Weber *et al.*, 1989), tyrosine plays an important part in the binding site of streptavidin by forming a critical hydrogen bond with the carbonyl carbon atom of biotin. The importance of the single tyrosine residue of egg-white avidin to the binding of biotin was corroborated by its selective group-specific modification and consequent inactivation of binding (Gitlin *et al.*, 1990). The analogous tyrosine residue in streptavidin underwent similar modification. In contrast, rather than interacting chemically, tryptophan residues appear to contribute more to the stabilization of the biotin-binding pocket, thereby securing the orientation of other amino acid residues that interact directly with the biotin molecule. Modification of tryptophan residues (Green, 1975; Gitlin *et al.*, 1988*a*,*b*) destroys the delicate architecture of the biotin-binding site in both proteins. It was therefore of interest to prepare biotin-binding fragments of avidin and/or streptavidin that would comprise the individual regions of the given protein suspected of contributing to the recognition of the vitamin.

Owing to the stability of avidin and streptavidin towards enzymic digestion, chemical methods had to be employed for the production of avidin fragments. With regard to hydroxylamine treatment, which is considered to be specific for Asn–Gly bonds, the sensitivity and resolution qualities of SDS/PAGE and blotting enabled us to detect new (and, in some cases, very minor) sites of cleavage on avidin. Most notable is the very rapid cleavage of the Asn–Glu bond (position 42–43) and perhaps the Asp–Lys bond (position 57–58), both of which appear to be cleaved much faster and even more specifically than the Asn–Gly bond. Such cleavage could eventually prove useful for the mild processing of genetically engineered proteins that contain either an *N*-terminal glutamic acid or an *N*-terminal lysine residue. In retrospect, the finding that the Asn–Glu bond is readily cleaved by hydroxylamine treatment is not surprising in view of the discovery that this bond is cleaved spontaneously in Nature (Voorter *et al.*, 1988).

The production of avidin fragments served to undo the structure of the biotin-binding pocket, which caused a drastic decrease in biotin binding. Consequently we were forced to develop new methodologies to detect such low-affinity interactions. The capacity to detect binding activity in individual fragments allowed us to distinguish between the contributions of the structural elements of the biotin-binding pocket and the chemistry of the bonds formed between the relevant amino acid residues of the protein and its substrate.

Since attempts to isolate active fragments by conventional methods were unsuccessful, we concentrated on the identification

and characterization of both active and inactive fragments on PVDF membranes after SDS/PAGE. This strategy was appealing for a number of reasons: (i) the strict isolation of individual fragments is not required to prove the activity of a given fragment; the activity of electrophoretically separated components in the mixture is determined *in situ* on the blot; (ii) 'isolated' fragments may also contain low concentrations of intact subunit, which would give 'false' positive results for biotin-binding; (iii) by using a more conventional approach, harsh conditions (which are sometimes required to isolate or solubilize a given peptide fragment) may inactivate biotin-binding ability. In contrast, the strategy employed here enabled the initial identification of the active bands, followed by subsequent characterization of the blotted fragment(s) associated with the desired band.

In using this approach, we compared the biotin-binding activity of fragments prepared from glycosylated versus non-glycosylated avidin. On the basis of the difference in M_r values of the active biotin-binding fragments, we could conclude that the active hydroxylamine-generated fragment is apparently one derived from the *N*-terminus of the protein. All of the unglycosylated fragments (i.e. residues 43–88, 58–104, 89–128 etc.) derived from native avidin appear not to exhibit biotin-binding activity on their own, since their relative mobilities would be unaltered after deglycosylation.

The major biotin-binding fragment produced upon hydroxylamine treatment of avidin was identified to include residues 1–42. The smallest active CNBr-cleavage fragment was that which comprised residues 19–96. This suggested that the most active biotin-binding region would reside between residues 19 and 42. This is confirmed by the binding activity observed for the synthetic tridecapeptide (residues 26–38) and the lack of activity exhibited by the synthetic peptide comprising residues 1–19. Strikingly, the active peptide included the tyrosine-containing stretch that is shared by both avidin and streptavidin, once again underscoring the primary importance of this domain for the recognition of biotin by these proteins. The data also imply a lesser role for the relevant amino acid residues in domain I (located in residues 1–19 of avidin) that are known to interact with biotin in the binding site (see Weber *et al.*, 1989; Wilchek & Bayer, 1989; Bayer & Wilchek, 1990*a*).

In light of these findings we could not ignore the fact that the fragment 19–42 (presumably obtained by the sequential CNBr–hydroxylamine treatment but not specifically identified because of the complexity of reaction products) appeared not to be active. Several reasons could account for the apparent lack of binding by this peptide. First of all, we cannot establish how much of this particular fragment was actually produced by the sequential treatment. Secondly, for reasons outlined above, we used different assay systems to analyse the binding capacity of avidin fragments versus that of synthetic peptides; we monitored the activity of the avidin fragment(s) in SDS/PAGE, using a sequential biotin-binding assay on blots, whereas the activity of the synthetic peptides (notably residues 26–38) was examined by a competitive binding assay employing radioactive streptavidin as a probe. Indeed, the synthetic peptide also failed to react in the more direct biotin-binding assay, which again may be related either to difficulties in the blotting of such a small peptide or in its sequential interaction with macromolecules while immobilized onto blots.

The finding that the synthetic 48-residue fragment of streptavidin (residues 77–124, a sequence that includes all of the tryptophan residues known to have a role in the biotin-binding site) failed to bind biotin also tends to support the above contention concerning the nature of the contribution of tryptophan and tyrosine residues. Our experimental data suggest that fragments derived from egg-white avidin that commence after residue 43 would be inactive. By analogy, we assume that streptavidin fragments commencing after residue 54 would also be inactive.

Finally, this study demonstrates experimentally that by using sensitive methods one can distinguish between the structural and chemical contributions of a protein–ligand interaction. The classical approach for examining the chemistry of a binding site involves the combined selective modification of individual amino acid residues on a protein and the assessment of whether the modification is accompanied by a loss in binding. Likewise, site-directed mutagenesis is also incapable of distinguishing between the structural and chemical elements of a binding site. These approaches are not always valid, since a modification may cause spatial distortion of the binding cavity, thus preventing high-affinity binding. In fact, this may be the case with avidin; the modification of tryptophan residues that leads to a loss in binding may reflect a simple destruction of the fine structure of the binding cavity, whereas the major chemical contribution is mainly restricted to the small tyrosine-containing fragment. The fine tuning between the structural and chemical elements together form the strongest binding between a protein and a ligand.

This research was supported by the Fund for Basic Research administered by the Israel Academy of Sciences and Humanities and by a grant from Makor Chemicals, Jerusalem, Israel.

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Received 12 December 1990/11 March 1991; accepted 13 March 1991