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Postsecretory modifications of streptavidin

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Streptavidin, an extracellular biotin-binding protein from *Streptomyces avidinii*, exhibits a multiplicity in its electrophoretic mobility pattern which depends both upon the conditions for growth of the bacterium and upon the protocol used in the purification of the protein. The observed structural heterogeneity appears to reflect the action of two types of postsecretory molecular events: proteolytic digestion of the intact M_r-18000 subunit to a minimal molecular size (approx. M_r 14000), and aggregation of the native tetramer into higher-order oligomeric forms. The extent of subunit degradation and/or tetrameric aggregation affects the capacity of a given streptavidin preparation to interact with biotin-conjugated proteins in different assay systems.

INTRODUCTION

Streptavidin is a non-glycosylated, neutral bacterial protein from *Streptomyces avidinii* which is remarkably similar in its biotin-binding properties to the positively charged egg-white glycoprotein, avidin. The strength of the binding affinity which these two proteins exhibit for biotin is the highest recorded between a protein and its ligand (Green, 1975). Despite the apparent lack of both immunochemical crossreactivity and evolutionary relatedness betwen the two proteins, several structural characteristics are shared. For example, both avidin and streptavidin are of similar molecular size and are composed of four subunits, each of which forms one biotin-binding site. Nevertheless, the amino acid composition and primary sequences are very different, although a series of short interrupted similar stretches are evident (Argarana *et al.*, 1986).

In addition to the fact that these biotin-binding proteins offer an intriguing model system for probing the interaction between a protein and its ligand (Green, 1975; Gitlin *et al.*, 1987, 1988*a*,*b*), the avidin–biotin complex provides an extremely useful, versatile system for general application in the biological sciences (Bayer & Wilchek, 1980; Wilchek & Bayer, 1984, 1988). Owing to its neutral pI and non-glycosylated structure, streptavidin has been used as a substitute in such systems, in order to circumvent problems arising from nonspecific binding which sometimes results from the use of egg-white avidin.

In recent studies, we (Bayer *et al.*, 1986*a*) and others (Argarana *et al.*, 1986) have demonstrated that the actual molecular mass of streptavidin is higher than that reported earlier (Chaiet & Wolf, 1964; Hofmann *et al.*, 1980). It appears that, either during growth or during the isolation process, the subunit of streptavidin may have been partially cleaved in some manner. In our initial work, we also noted a tendency for aggregation of the streptavidin tetramer. In the present communication, these two phenomena are further characterized.

MATERIALS AND METHODS

Streptavidin preparations

The commercially prepared streptavidin samples used in this work were kindly provided as gifts by Apcel Ltd. (Slough, Berks., U.K.), Boehringer-Mannheim G.m.b.H. (Mannheim, Germany), Calbiochem AG (Lucerne, Switzerland), Cetus Corporation (Emeryville, CA, U.S.A.), Immuno-search Inc. (Toms River, NJ, U.S.A.), Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, U.S.A.), Serotec (Kidlington, U.K.), Sigma Chemical Co. (St. Louis, MO, U.S.A.) and Zymed Laboratories Inc. (San Francisco, CA, U.S.A.). We received samples of several milligrams from Apcel, Sigma and Boehringer which enabled us to study their respective *N*- and *C*-terminal sequences.

Streptavidin was also prepared in our laboratory by growing 1–10 litre cultures of *S. avidinii*, and by isolating the protein from the spent growth medium using an improved iminobiotin–Sepharose affinity column as described previously (Bayer *et al.*, 1986*a*). Lot numbers referred to in the text indicate the product purified from a given fermentation run. Truncated forms of the protein were prepared by subjecting purified samples to extracts of the culture medium or to purified proteases as described below.

Preparation of whole-cell sonic extracts

S. avidinii was grown in liquid culture (100 ml) at 30 °C in medium A (Stapley *et al.*, 1963). The cells were harvested after 24 h and 96 h of growth, and the respective cell samples were washed three times with phosphate-buffered saline, pH 7.4 (PBS), by centrifugation (15000 *g*, 15 min). The washed cells were resuspended in 15 ml of PBS, homogenized, and sonicated in an ice bath (4 × 1 min at 50 W power; Branson Sonic Power Co., Danbury, CT, U.S.A.). Protein concentration was determined (Bradford, 1976), and the sonicated suspension was stored at −20 °C.

Abbreviations used: AS_{50} extract, 50 %-saturated-$(NH_4)_2SO_4$ fraction of cell-free culture fluids; e.l.i.s.a., enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline, pH 7.4; PAGE, polyacrylamide-gel electrophoresis.

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$(NH_4)_2SO_4$ fractionation of cell-free culture fluids

A 5-day cell culture (2 litres) was harvested, and the cell-free culture medium was collected. Half (1 litre) was dialysed exhaustively and lyophilized. The other half was treated with increasing increments of $(NH_4)_2SO_4$; all steps in the following protocol were carried out in ice or at 4 °C.

No precipitate could be detected in solutions containing up to 40%-saturated $(NH_4)_2SO_4$. At 50%-saturated $(NH_4)_2SO_4$, an ample amount of precipitate formed, which, after a 30 min incubation period, was collected by centrifugation, resuspended in a minimal volume of double-distilled water and saved. To the supernatant fluids, solid $(NH_4)_2SO_4$ was added to obtain a 60%-saturated solution, and the latter was stored overnight. The resultant precipitate was collected, and the above procedure was repeated successively to obtain 70%- (60 min incubation), 80%- (overnight incubation) and 100%-saturated (4 h incubation) $(NH_4)_2SO_4$ fractions. The precipitates were each dissolved in distilled water, dialysed exhaustively against distilled water and then once against PBS. Following dialysis, the residual precipitate in each sample was centrifuged and discarded. The respective protein concentrations were determined (Bradford, 1976), and the samples were stored in aliquots at −20 °C.

Treatment of streptavidin with cell sonic extracts and with $(NH_4)_2SO_4$ fractions

To 1 mg/ml samples of streptavidin in PBS (0.01% sodium azide added when required), the desired extract or fraction was added. Typically, unless otherwise indicated, 200 μg of the respective extract was added in 200 μl. The interaction was allowed to proceed at room temperature (unless otherwise stated). Aliquots (200 μl) were taken at various time periods (e.g., after 1, 3, 7 and 14 days of incubation) and stored at −20 °C pending SDS/polyacrylamide-gel electrophoresis (SDS/PAGE) analysis.

Treatment of streptavidin with proteases

All proteases mentioned in the text were purchased from Sigma. To 100 μg samples of streptavidin dissolved in 90 μl of an appropriate buffer (according to the manufacturer's suggestion for each protease), a solution (10 μl) containing 2 μg of the desired protease was added. 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, aliquots (50 μl) were removed, combined with 25 μl of sample buffer, boiled for 10 min and stored at −20 °C pending SDS/PAGE analysis.

SDS/PAGE and blotting

The conditions for SDS/PAGE and electrophoretic transfer on to nitrocellulose filters were described previously (Bayer *et al.*, 1986*a*; Hiller *et al.*, 1987). As described earlier (Bayer *et al.*, 1986*a*), the quaternary state of the streptavidin can be analysed by differential treatment of the samples with sample buffer. In the presence of SDS at relatively low temperatures (< 60 °C), the tetrameric (or oligomeric) form of the molecule is maintained. Above this threshold temperature, the molecule dissociates into its component parts. Upon boiling in SDS, most samples are generally reduced to their monomer form(s). Thus, in order to analyse the subunit composition of a given streptavidin sample, the samples were boiled in sample buffer and subjected to SDS/PAGE on 15% gels. For analysis of the oligomeric state of a given streptavidin sample, samples were treated in sample buffer at room temperature and run on 10% gels.

A combination of kits containing molecular mass markers (Sigma) were used as standards: MW-SDS-200 and MW-SDS-70L for the oligomer system; MW-SDS-70L and MW-SDS-17 for the monomer system.

Biotin-binding assays

In order to determine the biotin-binding capacity of a given streptavidin sample, two different assay systems were employed. In one, samples in solution were examined by an e.l.i.s.a.-like assay system which exploited the multivalent nature of the streptavidin molecule. In the second approach, the relative biotin-binding capacity of individual SDS/PAGE-separated bands was determined.

E.l.i.s.a.-like assay. Serial dilutions of streptavidin samples were incubated in wells of microtitre plates precoated with biotinyl bovine serum albumin. The biotin-binding activities of the streptavidin samples were then examined by interaction with biotinyl alkaline phosphatase as reported in a previous publication (Bayer *et al.*, 1986*b*).

Biotin-binding of streptavidin components on blots. The relative biotin-binding capacity of streptavidin monomers or oligomers was detected on nitrocellulose blots following SDS/PAGE separation. The blotted bands were treated with biotinyl alkaline phosphatase as described by Hiller *et al.* (1987). The signal associated with streptavidin oligomers usually appeared within 30 min; samples separated into monomeric components were usually incubated for 24 h periods after introduction of the substrate solution.

Gel chromatography

Gel filtration of streptavidin samples (2 mg in 1 ml) was carried out at 4 °C on a Sephacryl S-300 column, equilibrated and eluted with 50 mM-Tris/HCl buffer, pH 7.5, at a flow rate of 10 ml/h. The column dimensions were 15 mm × 670 mm, and 1.0 ml fractions were collected.

The desired fractions were pooled, dialysed against distilled water, and concentrated by lyophilization. The samples were stored at −20 °C at a concentration of 1 mg/ml.

Sequence analyses

The *N*-terminal sequence of a given streptavidin preparation was determined by Edman degradation using a gas-phase sequencer (three to five cycles per sample). The *C*-terminus was identified using carboxypeptidase Y according to Hayashi (1977). The *C*-terminal amino acid was confirmed by hydrazinolysis (Fraenkel-Conrat & Tsung, 1967).

RESULTS

Truncation of streptavidin subunits

Since our recent description of an improved method for purifying streptavidin, we have used the same

iminobiotin–Sepharose column and protocol for isolating over a dozen different lots which have collectively yielded more than 1.5 g of the protein. Upon SDS/PAGE under denaturing conditions, most of the lots were characterized by a major band at about M_r 18000 (Bayer *et al.*, 1986*a* and Fig. 1, lane a). In some lots, relatively low levels of one or more lower molecular mass bands often accompanied the major band. In one lot, Lot 5 (Fig. 1, lane b), the isolated product exhibited two major bands of lower molecular mass. Most commercially available preparations of this protein had an even more rapid mobility pattern on SDS/PAGE, but were usually characterized by a single band under denaturing conditions (Fig. 1, lanes c, d and e).

Initially, we were interested in determining whether the observed differences in relative molecular mass could be accounted for by an agent derived from the culture of *S. avidinii*, or whether an autolytic mechanism or external agent was responsible for the altered SDS/PAGE pattern (Arnold *et al.*, 1987). Samples of streptavidin (which were characterized by the major M_r-18000 band) were therefore treated for extended time periods either alone or in the presence of bacterial extracts (from the spent growth medium or from whole-cell sonicates); in some samples (as indicated below) sodium azide was added to prevent bacterial growth.

It can be seen from Fig. 2 that, in the absence of azide, extensive degradation of the M_r-18000 band takes place without additional additives. The observed degradation was pH- and temperature-dependent, showing broad optima at neutral pH and ambient temperatures, reaching completion after 2–4 days of incubation. Incubation of streptavidin at 4 °C or at 42 °C and at pH extremes (< 3, $\geqslant 10$) served to counter the alteration in molecular mass of the streptavidin subunit. In addition, this effect could be eliminated by passing streptavidin samples through a sterilized cellulose acetate filter (0.45 μm). Microscopic examination of streptavidin samples after extended incubation periods at room temperature confirmed the presence of microbial contaminants.

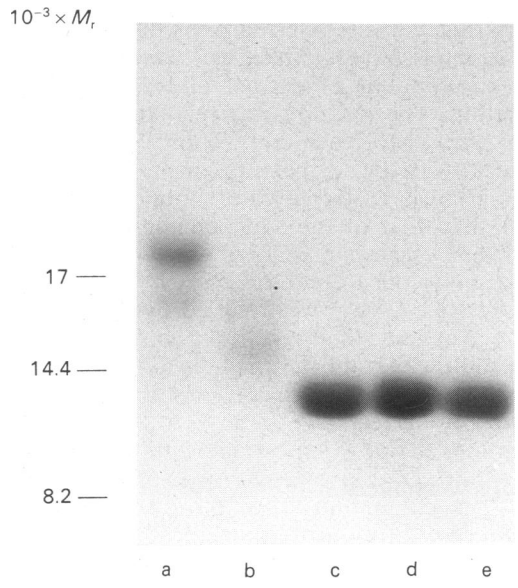

Fig. 1. Characteristic subunit mobility patterns of selected streptavidin preparations

Laboratory samples of streptavidin from Lots 7 and 5 (lanes a and b respectively) and commercial preparations from Apcel, Sigma and Boehringer (lanes c, d and e respectively) were subjected to SDS/PAGE (15% gel) under denaturing conditions (boiling of samples).

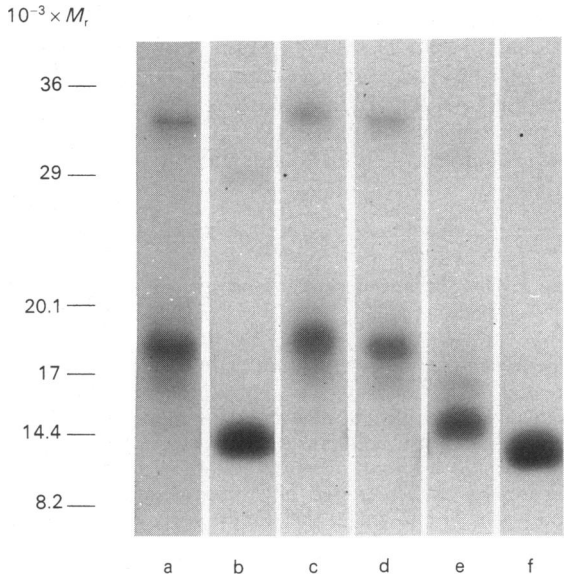

Fig. 2. Partial degradation of purified streptavidin subunit by external agents

A streptavidin sample (Lot 7), which exhibits high levels of the intact M_r-18000 subunit, was subjected to the following treatments: lane a, none (freshly prepared sample applied to gel); lane b, incubation for 4 days in the absence of azide; lane c, incubation for 14 days in the presence of azide; lane d, incubation for 14 days in the presence of whole-cell sonicate from 96 h culture of *S. avidinii*; lane e, incubation for 14 days in the presence of cell-free growth medium (in the presence of azide). Following the latter treatments, the samples were analysed by SDS/PAGE under the conditions used in Fig. 1. Lane f shows the relative position of Apcel streptavidin. Note the retention of dimers in some of the samples.

In the presence of azide, however, the integrity of the M_r-18000 streptavidin band was maintained for a period of at least 3 weeks at room temperature. Other bactericidal or bacteriostatic agents, including hydrazine, cycloheximide, actinomycin, streptomycin and SDS, could replace sodium azide in preventing the degradation of streptavidin subunits (results not shown). Likewise, incubation of streptavidin (in the presence of azide) with whole-cell sonicates of *S. avidinii* (derived from cells grown for either 24 h or 72 h) also failed to affect the mobility pattern of the streptavidin subunit. This indicates that a cell-associated agent is not responsible for the observed degradation of streptavidin. In contrast, incubation of streptavidin with extracellular material (derived from the cell-free growth medium) in the presence of azide led to a slow but steady incremented decline in the molecular mass of the streptavidin subunits (Fig. 2, lane e).

In order to examine further the nature of the extracellular material which caused this effect, we fractionated the cell-free growth medium using graded concentrations of $(NH_4)_2SO_4$ solutions. Below 50% saturation, $(NH_4)_2SO_4$ failed to precipitate detectable amounts of material from the growth medium. Above

this level, measurable quantitites of proteins could be extracted (Table 1). SDS/PAGE (Fig. 3) revealed that each of the fractionated samples contained a large number of protein bands. With respect to streptavidin, the majority was precipitated at 60% saturation of $(NH_4)_2SO_4$; relatively little was detected at 50% saturation.

Table 1. Distribution of protein and streptavidin in $(NH_4)_2SO_4$ fractions

Spent, cell-free culture medium (5 days growth, 1 litre) was treated with increasing concentrations of $(NH_4)_2SO_4$ as indicated. The amount of streptavidin and protein content of the resultant fractions were determined. Purity refers to the specific content of streptavidin in a given sample (expressed as percentage of streptavidin relative to the total protein content in the sample).

$(NH_4)_2SO_4$ (% saturation)	Total protein (mg)	Streptavidin (mg)	Purity (%)
0	212.0	13.7	6.5
50	49.0	1.3	2.7
60	76.0	12.4	16.3
70	4.5	0.9	19.1
80	12.6	0.5	3.8
100	16.0	0.4	2.2

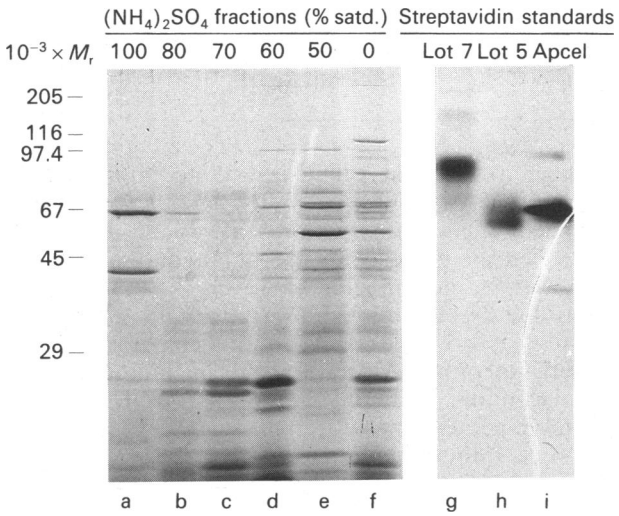

Fig. 3. SDS/PAGE pattern of extracellular *S. avidinii* proteins precipitated by various saturated solutions of $(NH_4)_2SO_4$

Samples of the cell-free growth medium were treated with the indicated (% saturation) concentrations of $(NH_4)_2SO_4$, and the solubilized precipitate was subjected to SDS/PAGE (10% gel, unboiled samples). 0% indicates the untreated (dialysed and concentrated) cell-free culture fluids. In lanes a, b and c, 100 μg of protein was applied; in lanes d, e and f, 200 μg of protein was applied. The relative positions of streptavidin are given for comparison (under non-denaturing conditions). Note that, unlike the behaviour under denaturing conditions (Fig. 1), Apcel streptavidin is characterized by several bands (note the faint dimer and hexamer band in addition to the native tetramer).

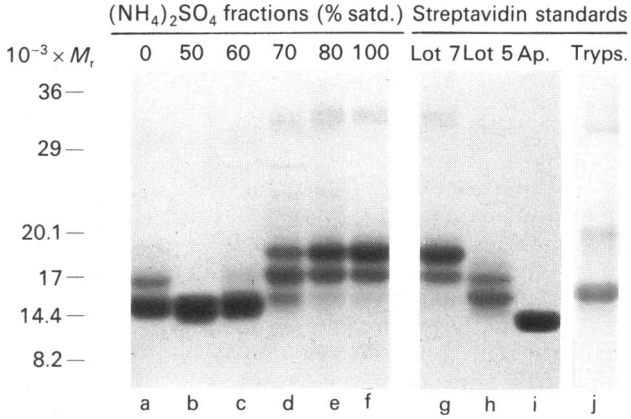

Fig. 4. Degradative activity of different $(NH_4)_2SO_4$ fractions

A 200 μg sample of streptavidin, dissolved in 200 μl of PBS (in the presence of 0.01% sodium azide), was treated with an aliquot (40 μg in 13.3 μl) of the indicated (%-saturated) $(NH_4)_2SO_4$ precipitate of the cell-free culture fluids. After a 14 day incubation period (in the presence of azide), the relative mobility on SDS/PAGE of each sample was compared to that of the starting material (Lot 7, lane g). The mobilities of Apcel (Ap.), Lot 5 and trypsin-treated (Tryps.) streptavidin are shown for comparison. Lane a shows the action on streptavidin of the untreated (dialysed, concentrated) cell-free culture fluids. In all lanes, 30 μg of protein was applied.

As can be seen from Fig. 4, at $(NH_4)_2SO_4$ concentrations of 70% saturation and above, little or no degradative activity of the M_r-18000 streptavidin band could be observed. The most pronounced effect was evident at 50% saturation, with somewhat diminished activity at 60%.

Using the 50%-saturated $(NH_4)_2SO_4$-precipitable material, hereinafter referred to as the AS_{50} extract, the truncation of streptavidin subunits was found to depend upon the amount of extract added and the time of incubation. The reaction was relatively slow, requiring up to 2 weeks to reach completion. Significantly, upon treatment with the AS_{50} extract, the final molecular mass of the truncated streptavidin subunit was measurably higher than that of the commercially available protein (e.g. from Apcel or Sigma) as well as that obtained by incubation in the absence of azide. A broad spectrum of reaction conditions was observed upon treatment with the AS_{50} extract. The reaction proceeded to apparent completion within the pH range 3–11 (the reaction failed to go at pH 2.2 and pH 12). Permissible temperatures ranged from as low as 4 °C up to at least 42 °C (2 week incubation period). Upon incubation for 10 min at 60 °C, the degradative activity was preserved; however, incubation for 10 min at 80 °C destroyed the activity. The presence of iodoacetic acid reduced the rate of degradation somewhat, but a variety of other protease inhibitors, including phenylmethanesulphonyl fluoride, *N*-tosyl-phenylalanine chloromethyl ketone, N^{α}-tosyl-lysine chloromethyl ketone, *N*-ethyl maleimide, soybean trypsin inhibitor, leupeptin and pepstatin failed to affect the reaction.

The M_r-18000 streptavidin subunit was sensitive to the action of commercially available proteolytic enzymes. Proteinase K, papain, protease (from *Streptomyces*

griseus), subtilisin, thermolysin and elastase (each at a 1:50 w/w protease/substrate ratio) successfully converted streptavidin into truncated forms, the mobility of each on SDS being very similar to that of the commercially available protein. The proteolysis proceeded very rapidly, essentially reaching completion in most cases within a 15 min incubation period. Trypsin also degraded streptavidin, but the position of the product on SDS/PAGE (Fig. 4) was measurably higher than that of either the commercial preparations or the AS_{50}-treated form. Chymotrypsin and pepsin (2 h incubation period) failed to degrade the native (M_r-18000) streptavidin subunit. Under the conditions used in this study, none of the commercially obtained proteases succeeded in degrading the native subunit to a molecular mass smaller than that of the commercial preparations of streptavidin, nor were any active on the latter preparations.

Cleavage sites of truncated streptavidin

Comparative *N*- and *C*-terminal analyses of the various laboratory and commercial streptavidin preparations revealed the following information (see Fig. 5 for summary). The major component of most of our laboratory preparations (excluding Lot 5) is the intact gene product (following prior cleavage of the leader sequence upon export). The major component both of the Apcel and Boehringer preparations is that given by Pähler *et al.* (1987). This differs slightly from Sigma streptavidin, the major component of which bears Glu-14 at its *N*-terminus with the remainder starting from Ala-13. Hydrazinolysis demonstrated that serine is the major *C*-terminal amino acid in all of the commercial preparations tested.

The cleavage sites of the major AS_{50}-treated truncated streptavidin product differed from those of the commercial preparations. The results indicated one or two extra amino acids at the *N*-terminus and two additional residues at the *C*-terminal end. Thus, this form of streptavidin appears to be cleaved between Ser-11 and Ala-12 at the *N*-terminus and between Asp-141 and Ala-142 at the *C*-terminus. These results are consistent with the SDS/PAGE behaviour of the respective proteins (Figs. 2 and 4); i.e. the SDS/PAGE mobility of the commercial forms is faster than that of the AS_{50}-treated protein. As would be expected from its presumed sites of cleavage [the Lys-7–Ala-8 bond at the *N*-terminus and the Lys-144–Lys-145 (or Lys-145–Ala-146) bond at the *C*-terminus], trypsin-treated streptavidin migrates even slower than the AS_{50}-treated product (Fig. 4). It is also interesting to note that the failure of chymotrypsin and pepsin to act on the *N*- and *C*-termini is consistent with the absence of aromatic amino acids within these protease-sensitive terminal stretches.

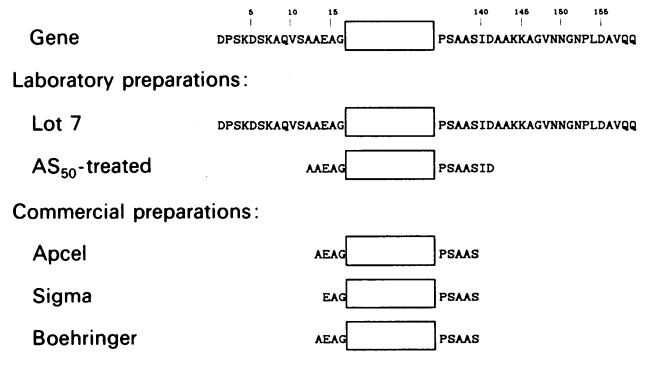

Fig. 5. Composition of the terminal sequences of various streptavidin preparations

The sequences shown represent those of the major component of the designated preparation and were determined experimentally by *N*- and *C*-terminal analyses. The gene sequence is that given by Argarana *et al.* (1986). The intervening residues betwen Gly-16 and Pro-135 have been omitted for clarity and are designated by the elongated boxes.

Aggregation of streptavidin tetramers

In many of the preparations which were isolated in our laboratory, higher-order aggregates were often observed upon SDS/PAGE using conditions under which the tetrameric structure of streptavidin is maintained (unboiled samples, 10% gels). This was especially noticeable in one particular lot (Lot 4) of streptavidin, and thus raised the question of whether these aggregates normally exist in solution or whether their formation is a function of the electrophoretic conditions (i.e. SDS etc.).

We therefore tried to separate higher-order aggregates from single tetramers by subjecting a solution of streptavidin Lot 4 to gel filtration on a Sephacryl S-300 column (Fig. 6). In addition to the relatively broad peak representing the free tetramer, a second, smaller peak could be discerned. Eluted fractions were pooled as illustrated in Fig. 6, and the concentrates (I–V) were analysed by SDS/PAGE. It is clear from Fig. 7 that the initial peak (I) contained enriched fractions of streptavidin oligotetramers. Di-, tri- and tetra-tetramers could be discerned. Analysis of such higher-order aggregates in denaturing gels (boiled samples) revealed the presence of correspondingly high levels of subunit dimers (results not shown). This suggests that intermolecular cross-linking (connecting perhaps between terminal stretches of aggregated molecules) may account for the formation of oligotetrameric species of streptavidin.

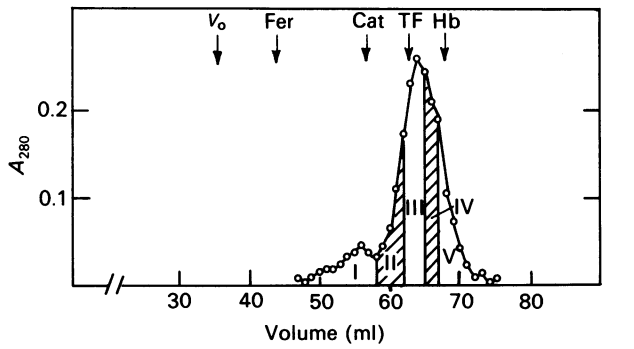

Fig. 6. Gel filtration pattern of an oligomer-enriched preparation of streptavidin

Streptavidin Lot 4 (2 mg in 1 ml) was applied to a Sephacryl S-300 column. The eluted fractions were pooled as indicated by roman numerals I to V. Arrows indicate the elution positions for the void volume (V_o), ferritin (Fer), catalase (Cat), transferrin (TF) and haemoglobin (Hb).

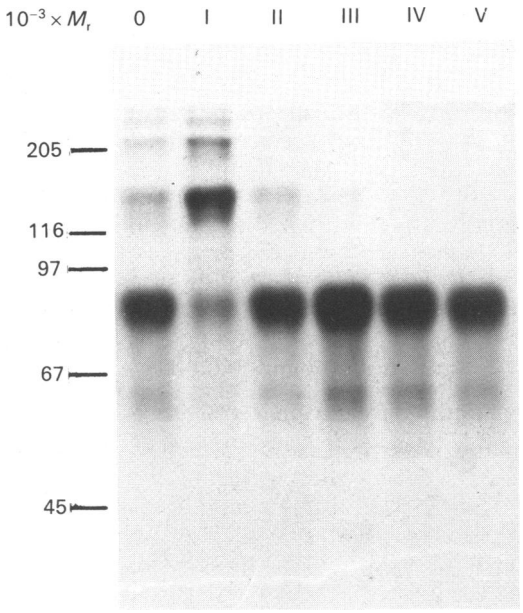

Fig. 7. Enrichment of streptavidin oligomers

The pooled, eluted fractions from the Sephacryl S-300 column shown in Fig. 6 were concentrated, and 30 μg samples were analysed by SDS/PAGE under non-denaturing conditions (unboiled samples, 10 % gel). Note the highly enriched concentration of oligotetramers in fraction I.

Effect of culture age

Four 3-litre cultures of *S. avidinii* were harvested after growth at 30 °C for various time periods (2, 5, 9 and 13 days). Streptavidin was isolated from each sample by affinity chromatography on an iminobiotin–Sepharose column, and the characteristics of the respective isolates were analysed by SDS/PAGE. The results (not shown) indicated that both the limited proteolysis of the streptavidin subunit and the tendency to aggregate are more pronounced at the later stages of growth. The relative extent of truncation and/or aggregation varied greatly between different fermentation runs. The factor(s) responsible for induction or repression of either of these phenomena have yet to be identified.

Biotin-binding activity of streptavidin preparations

In order to test the relative activity of the various streptavidin preparations, several assay procedures were employed, based generally on the reaction of streptavidin with biotin-labelled alkaline phosphatase.

In one approach, the desired streptavidin sample was incubated with biotinylated (biotinyl albumin-coated) microtitre plates, and the unoccupied biotin-binding sites on the adsorbed streptavidin molecules were subsequently subjected to interaction with biotinyl alkaline phosphatase. The level of enzyme activity was then determined in an e.l.i.s.a.-like procedure using a microtitre plate reader (Bayer *et al.*, 1986*b*). Using this procedure, the various laboratory and commercial preparations exhibited surprisingly different levels of binding activity for biotinyl proteins (Table 2). Most strikingly, the binding of Apcel streptavidin was almost 8-fold higher than that of our reference sample. Since the major molecular difference between these samples is the truncated form of the commercial preparation versus the intact form of the laboratory preparation, we questioned whether a similar effect could be achieved by preparing the truncated form of streptavidin *in vitro*. The reference sample (Lot 7) was therefore treated with the AS_{50} extract in the presence of azide for extended periods of time. Assaying the 7-day and 14-day samples for biotin binding by the e.l.i.s.a.-like system clearly showed a marked increase in activity (Table 3), suggesting that the truncated form of streptavidin was enhanced in its interaction with biotinylated proteins. Using this system, aggregated forms of streptavidin (e.g. Lot 4) showed relatively low activity levels in binding biotinyl alkaline phosphatase (Table 2).

In contrast to these results, in a second approach (following Western blotting of SDS/PAGE samples) reduced binding of biotinyl alkaline phosphatase was observed for the truncated form of streptavidin (Fig. 8). This was true both for samples which retained their tetrameric structure (unboiled, 10 % gels) and for subunit-resolved samples (boiled, 15 % gels). Moreover, under these same conditions, streptavidin oligomers clearly exhibited higher levels of reactivity with biotinyl

Table 2. Relative interaction of various streptavidin preparations with biotinyl alkaline phosphatase

The interaction of various laboratory preparations of streptavidin was compared with that of a commercially supplied truncated form (from Apcel) by subjecting the indicated samples to the e.l.i.s.a-like assay procedure (Bayer *et al.*, 1986*b*). Lot 7 was used as a reference. Lot 4, fraction I refers to the chromatographically separated, oligotetramer-enriched preparation (see Figs. 6 and 7).

Streptavidin preparation	Relative activity
Lot 7	1.00
Lot 4	0.55
Lot 4, fraction I	0.50
Lot 5	1.16
Apcel	7.67

Table 3. Effect of AS_{50} extract on binding activity

Samples of streptavidin (Lot 7) were incubated with azide for the given time periods either in the presence or in the absence of AS_{50} extract as indicated. The interaction with biotinylated proteins was examined by the e.l.i.s.a.-like assay.

Sample	Incubation period (days)	Relative activity
Streptavidin	0	1.00
Streptavidin	7	1.24
Streptavidin	14	0.90
Streptavidin + AS_{50} extract	0	1.19
Streptavidin + AS_{50} extract	7	4.73
Streptavidin + AS_{50} extract	14	5.59

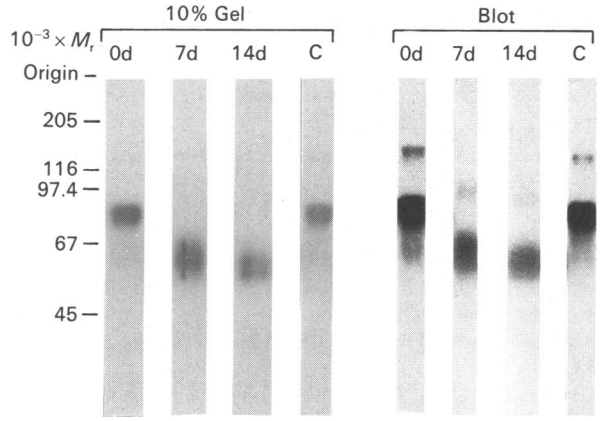

Fig. 8. Effect of AS_{50} extract on binding activity between streptavidin and biotinyl alkaline phosphatase following SDS/PAGE and Western blotting

Samples of streptavidin (Lot 7) were treated with the AS_{50} extract (with azide) for the indicated time periods (d, days). The unboiled samples (30 μg) were subjected to SDS/PAGE (10% gels) and either stained with Coomassie Blue (gel) or blotted on to nitrocellulose filters (blot). The blot was treated with biotinyl alkaline phosphatase and substrate solution. The mobility pattern of the untreated protein (C) is shown for comparison. Note the relatively weak staining pattern of the blotted 14 day sample; di-tetramers, visible in the initial sample, were markedly reduced in samples treated for 7 and especially 14 days. Essentially the same pattern was observed for commercial preparations (Apcel, Sigma, etc.) of streptavidin as for the 14 day sample. Likewise, under denaturing conditions (boiled samples, 15% gels), samples treated with AS_{50} extract for 7 and 14 days exhibited reduced reaction with biotinyl alkaline phosphatase compared to the untreated and 0 day samples (not shown).

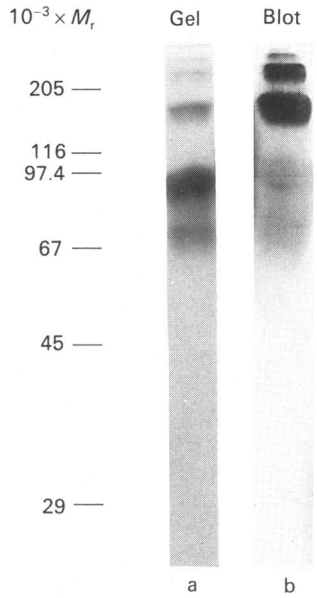

Fig. 9. Enhanced biotin-binding activity of streptavidin oligotetramers on blots

Samples (30 μg) of the oligotetramer-enriched streptavidin preparation (Lot 4) were subjected to SDS/PAGE (unboiled samples, 10% gel) and either stained with Coomassie Blue (lane a) or blotted and subjected to interaction with biotinyl alkaline phosphatase (lane b). Note the relatively high level of interaction of the oligotetramers compared to that of the unaggregated tetramer.

alkaline phosphatase compared to the tetramer (Fig. 9). The activity of a given streptavidin preparation thus appears to depend upon the state of its quaternary structure as well as on the assay system used.

DISCUSSION

The results of this study demonstrate that two major types of postsecretory modifications of the extracellular biotin-binding protein streptavidin occur during growth of *S. avidinii*. The bacterium appears to produce very low quantities of an extracellular protease(s), which selectively hydrolyses streptavidin at both the *N*- and *C*-terminal ends. In addition, the protein tends to form stable oligotetramers in solution.

It is interesting to note that in earlier works (Chaiet & Wolf, 1964; Hofmann *et al.*, 1980) $(NH_4)_2SO_4$ solutions at 70% saturation were employed to precipitate streptavidin prior to further purification. The results here show that under these conditions both streptavidin and the protease(s) responsible for truncation would be co-precipitated (and concentrated), thus enhancing the degradative effect. This can probably account for the relatively low values (approx. M_r-60000) reported for the molecular mass of streptavidin in the above-mentioned works.

The proteolytic capacity associated with *S. avidinii*, however, appears not to be responsible *in toto* for the level of degradation observed in both the commercially obtained preparations (e.g. Apcel, Sigma, etc.) and those subjected to incubation in the absence of bactericidal agents. It appears that, at least in the latter case, fortuitous microbial growth (and concomitant production of additional proteases) leads to the truncation of the native M_r-18000 subunit to a minimum size. The successful isolation of native (untruncated) streptavidin, as described herein and in our previous article (Bayer *et al.*, 1986*a*), appears to have hinged on the rapidity of the separation procedure which employs an improved iminobiotin–Sepharose column and precludes the use of $(NH_4)_2SO_4$ precipitation.

A commercial form of streptavidin (from Apcel), which had been reduced to a minimal size by an undisclosed process, has recently been characterized regarding its cleavage sites, solubility properties, crystallization and molecular symmetry (Pähler *et al.*, 1987). This type of streptavidin, which exhibits this minimal size, was termed 'core' streptavidin and was shown to consist of a moderately heterogeneous mixture of forms which had been cleaved at or near a repeating Ser-Ala-Ala sequence which appears at both ends of the molecule (positions 11–13 and 136–138). The lack of activity of commercial proteases on core streptavidin demonstrated in the present report underscores the remarkable stability of this form of the protein.

The failure of the AS_{50} extract to reduce streptavidin to this minimal size, however, implies that the cleavage site(s) of the protease(s) inherent to *S. avidinii* are different; an extra portion (one or two amino acid residues) remains on both the *N*- and *C*-terminal ends of the streptavidin molecule. Indeed, the ready accessibility

of the untruncated streptavidin termini to proteolytic action supports the suggestion of Pähler *et al.* (1987) that they are relatively flexible appendages on core streptavidin. In this context, the results presented in Tables 2 and 3 show that upon removal of these appendages, the interaction with biotinyl alkaline phosphatase is increased dramatically. This would suggest that in solution the appendages of the native intact molecule are positioned such that accessibility of the biotin moiety in conjugated systems is blocked.

With this in mind, it would appear surprising that following SDS/PAGE and Western blotting, the levels of interaction with biotinyl alkaline phosphatase displayed by streptavidin oligomers are much higher than that of the tetramer, which in turn is higher than that of the truncated molecule. These results are consistent with the notion that the oligotetrameric forms of streptavidin are structurally more stable to the electrophoretic procedure carried out before interaction with biotinyl alkaline phosphatase. This may suggest a stabilizing role for the terminal appendages in streptavidin.

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