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Studies on the biotin-binding sites of avidin and streptavidin

Tyrosine residues are involved in the binding site

Gerry GITLIN, Edward A. BAYER and Meir WILCHEK*
Department of Biophysics, The Weizmann Institute of Science, Rehovot 76100, Israel

The involvement of tyrosine in the biotin-binding sites of the egg-white glycoprotein avidin and the bacterial protein streptavidin was examined by using the tyrosine-specific reagent *p*-nitrobenzenesulphonyl fluoride (Nbs-F). Modification of an average of about 0.5 mol of tyrosine residue/mol of avidin subunit caused the complete loss of biotin binding. This indicates that the single tyrosine residue (Tyr-33) in the avidin subunit is directly involved in the biotin-binding site and that its modification by Nbs also abolishes the binding properties of a neighbouring subunit. This suggests that the tyrosine residues of the egg-white protein may also contribute to the stabilization of the native protein structure. In streptavidin, however, the modification of an average of 3 mol of tyrosine residue/mol of subunit was required to inactivate completely the biotin-binding activity of the protein, but only 1 mol (average) of tyrosine residue/mol of subunit was protected in the presence of biotin. The difference between the h.p.l.c. elution profiles of the enzymic digests of Nbs-modified streptavidin and the Nbs-modified streptavidin–biotin complex revealed two additional fractions in the unprotected protein that contain Nbs-modified tyrosine residues. These residues, Tyr-43 (major fraction) and Tyr-54 (minor fraction), appear to contribute to the biotin-binding site in streptavidin.

INTRODUCTION

The egg-white glycoprotein avidin and its non-glycosylated bacterial analogue streptavidin are two evolutionarily unrelated proteins that bind the vitamin biotin with remarkably high affinity constants (K_a approx. 10^{15} M^{-1}). This unique feature is the basis of avidin–biotin technology, which has evolved into a universal tool in various fields of the biological sciences (Bayer & Wilchek, 1978, 1980; Wilchek & Bayer, 1984, 1988, 1989, 1990).

In addition to biotin-binding, both proteins share many similar physical properties. In this regard, both are mainly constructed from β-pleated sheets and belong to the protein class containing orthogonal β-barrel structures, such as retinol-binding protein and β-lactoglobulin (Green, 1990). Avidin and streptavidin are characterized by similar M_r values (Green, 1975; Bayer *et al.*, 1986; Argarana *et al.*, 1986; Pähler *et al.*, 1987), and both are tetramers of identical subunits, each of which contains a single biotin-binding site. Although the amino acid compositions of the two proteins are very different, about 38% identity can be deduced from the two sequences, generally expressed as a series of similar short stretches rather than large domains.

Despite these similarities, some notable differences exist between these two proteins. Avidin is a glycoprotein and contains one disulphide bridge and two methionine residues, whereas streptavidin is non-glycosylated and devoid of any sulphur-containing residues. Whereas avidin bears a single tyrosine residue, the content of tyrosine residues in streptavidin is relatively high (six per subunit). Interestingly, the tyrosine residue in avidin (Tyr-33) is located in the primary sequence in a position similar to one of the tyrosine residues (Tyr-43) of streptavidin (Fig. 1). Moreover, this analogous tyrosine residue is located in the longest similar stretch found in both proteins.

	30	35
Avidin	Thr-Gly-Thr-Tyr-Ile-Thr-Ala-Val	
	40	45
Streptavidin	Thr-Gly-Thr-Tyr-Glu-Ser-Ala-Val	

Fig. 1. Conserved sequences of tyrosine-containing stretches in avidin and streptavidin

In previous work we have studied the involvement of lysine and tryptophan residues in biotin binding by avidin and streptavidin (Gitlin *et al.*, 1987, 1988*a*,*b*). We have also described some interesting spectral characteristics of tyrosine residues in both proteins (Gitlin *et al.*, 1989). The present work deals with the elucidation of the role played by tyrosine residues in the biotin-binding sites of avidin and streptavidin.

MATERIALS AND METHODS

Materials

Avidin was generously provided by Societé Cooperative Belovo (Bastogne, Belgium) and STC Laboratories (Winnipeg, Manitoba, Canada). Streptavidin was isolated from the broth of *Streptomyces avidinii*, and the native (untruncated) form was purified as described previously (Bayer *et al.*, 1986) with an improved iminobiotin–Sepharose column. (+)-Biotin, trypsin (L-tosylphenylalanylchloromethane-treated), chymotrypsin (L-tosyl-lysylchloromethane-treated), *p*-nitrobenzenesulphonyl fluoride (Nbs-F) and 4′-hydroxyazobenzene-2-carboxylic acid were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other reagents were of the highest available analytical grade.

Biotin-binding assays for avidin and streptavidin activity

Two biotin-binding assay methods were used interchangeably to assess undamaged biotin-binding sites after chemical modification of avidin. The first was based on the use of the dye 4′-hydroxyazobenzene-2-carboxylic acid (Green, 1970), which binds to avidin and can be removed by the addition of biotin. The binding of 4′-hydroxyazobenzene-2-carboxylic acid by avidin is accompanied by spectral changes (ϵ_{500} 35000 $M^{-1} \cdot cm^{-1}$), and the dye is stoichiometrically displaced upon interaction with biotin. The second assay was based on fluorimetric titration of biotin by the method of Lin & Kirsh (1977).

For streptavidin, only the fluorimetric assay for biotin binding

Abbreviation used: Nbs, *p*-nitrobenzenesulphonyl.

* To whom correspondence should be addressed.

was used, since the 4'-hydroxyazobenzene-2-carboxylic acid interacts poorly with streptavidin.

Formation of the avidin–biotin complex or streptavidin–biotin complex

A stock solution of protein (10 mg/ml) was diluted to the required concentration by adding the appropriate buffer. A sample containing 1.2 equiv. of biotin (stock solution 5 mg/ml in 0.15 M-NaCl/10 mM-sodium phosphate buffer, pH 7.4) was added to the protein solution and incubated for 5 min before use at room temperature.

Tyrosine modification

Avidin or streptavidin was modified by using the tyrosine-specific reagent Nbs-F (Liao *et al.*, 1982). In the case of avidin, the reaction was performed in 0.5 ml samples (3.1 mg of protein/ml) in 0.1 M-$NaHCO_3$ pH 7.9, with Nbs-F at ratios of reagent ranging from 0.5:1 to 32:1 with respect to the molar concentration of avidin subunit. The reaction was carried out for 20 h at room temperature. The solution was dialysed against water. Similar treatment was performed on the avidin–biotin complex.

In the case of streptavidin, the reaction was performed in 1 ml samples (1.4 mg/ml) with a 100-fold molar excess of the reagent with respect to the molar concentration of streptavidin subunit. Samples (0.1 ml) were extracted at different time intervals and the reaction was terminated by the addition of excess of phenol.

For preparative studies, a sample of streptavidin (1.6 mg) was dissolved in 0.2 ml of 0.1 M-$NaHCO_3$, pH 7.9. A 100-fold molar excess of Nbs-F with respect to streptavidin subunit was added, and the reaction was carried out for 20 h. Similar treatment was performed on the streptavidin–biotin complex. Modified samples were dialysed against water and freeze-dried. In each case the extent of Nbs modification was determined by amino acid analysis.

Tryptic hydrolysis

Nbs-modified streptavidin and Nbs-modified streptavidin–biotin complex (1.6 mg each) were each dissolved in 0.2 ml of 10 M-urea and boiled for 15 min. The samples were dialysed twice against 2 M-urea solution at 50 °C and three times against distilled water. This procedure was designed to remove biotin from the complex. The samples were then boiled again for 15 min, the pH was adjusted to 8 by the addition of 0.8 ml of 0.1 M-sodium bicarbonate buffer, pH 8.0, and the samples were hydrolysed by addition of 32 μg of trypsin for 20 h at 38 °C. Half of the sample (0.5 ml) was immediately subjected to chymotryptic hydrolysis (see below), and the other half was acidified by addition of 10 μl of 5% (v/v) trifluoroacetic acid and centrifuged.

Chymotryptic hydrolysis

The tryptic digest of the desired sample (0.8 mg) was subjected to 2 h chymotryptic hydrolysis by addition of 16 μg of enzyme at 38 °C.

Reverse-phase h.p.l.c. separation of peptide fragments

Samples (0.5 ml) containing the digested Nbs-modified protein were subjected to reversed-phase h.p.l.c. under the following conditions. A Lichrosphere Select B RP-8 (Merck) cartridge column (250 mm × 4 mm) was used for peptide separation; a 60 min linear gradient was initiated with 90% (v/v) of solvent A [0.1% (v/v) trifluoroacetic acid in distilled water] ending with 80% (v/v) of solvent B [0.1% (v/v) trifluoroacetic acid in 50% (v/v) propanol-1-ol]. The flow rate was calibrated at 0.5 ml/min. The corresponding fractions from the Nbs-containing peaks were collected; the amino acid composition and *N*-terminal sequence were determined.

Amino acid analysis

The amino acid composition of the desired sample was determined after hydrolysis of the samples in 6 M-HCl under vacuum at 110 °C for 22 h. The analyses were performed on a D550 Analyser (Durrum Instrument Corp., Palo Alto, CA, U.S.A.).

Sequence determination

Sequence analyses were carried out in an Applied Biosystems model 470A sequencer. Phenylthiohydantoins were identified by complementary h.p.l.c. systems.

RESULTS

The primary amino acid sequence of egg-white avidin contains a single tyrosine residue, located at position 33. This residue may be modified upon addition of a large excess of the tyrosine-specific reagent Nbs-F. The extent of tyrosine modification was determined by amino acid analysis. By means of this technique the specific interaction of the reagent with the tyrosine residue was established; other amino acid residues did not react with this reagent under the conditions described here.

Avidin was treated with various amounts of the reagents, and, correspondingly, different quantities of Nbs were incorporated into the protein. The biotin-binding activity of avidin decreased upon increasing the content of modified tyrosine. Statistical analysis of the results graphed in accordance with Tsou (1962) indicated that 0.5 mol of Nbs-modified tyrosine residue/mol of avidin subunit is required to inactivate completely the biotin-binding activity of the protein (Fig. 2). Hence modification of two tyrosine residues per avidin tetramer is sufficient to abolish biotin binding. The avidin–biotin complex, treated identically, is resistant to the reagent; no modified tyrosine was observed (Fig. 2 inset). The incorporation of more than 0.5 mol of reagent/mol of subunit became increasingly more difficult, probably as a result of conformational changes developed after introduction of the initial 0.5 mol of the reagent/mol (Fig. 2 inset).

A similar approach was applied to the bacterial protein streptavidin. At least three of the six tyrosine residues per

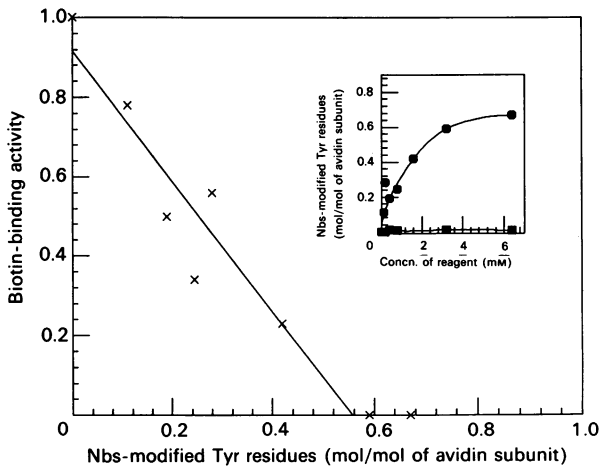

Fig. 2. Effect of the modification of tyrosine residues on the biotin-binding activity of avidin

The percentage of activity remaining after modification (compared with the activity of the native protein) is plotted against the number of modified tyrosine residues. Modification of an average of 0.5 mol of residue/mol of subunit incurred 100% loss in the activity. Inset: protection of biotin-binding site from Nbs modification. Solutions (0.5 ml, 0.2 mM in avidin subunits), containing either avidin (●) or the avidin–biotin complex (■), were treated with different concentrations (ranging from 0.1 to 6.4 mM, final) of Nbs-F.

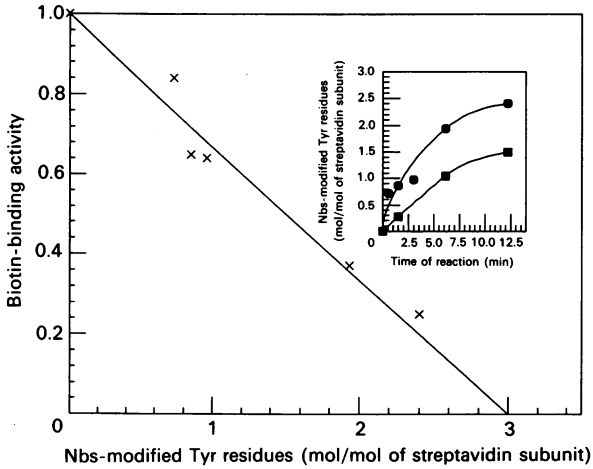

Fig. 3. Effect of modification of tyrosine residues on the biotin-binding activity of streptavidin

The percentage of activity remaining after modification (compared with the activity of the native protein) is plotted against the number of modified tyrosine residues. Modification of an average of 3 mol of residues/mol of subunit resulted in 100% loss in the activity. Inset: protection of biotin-binding site from Nbs modification. Solutions (1.0 ml, 0.1 mM in streptavidin subunit), containing either free streptavidin (●) or the streptavidin–biotin complex (■), were treated with a 100-fold excess of reagent with respect to the streptavidin monomer. Samples (0.1 ml) were extracted at different time intervals, and the reaction was terminated by the addition of an excess of phenol.

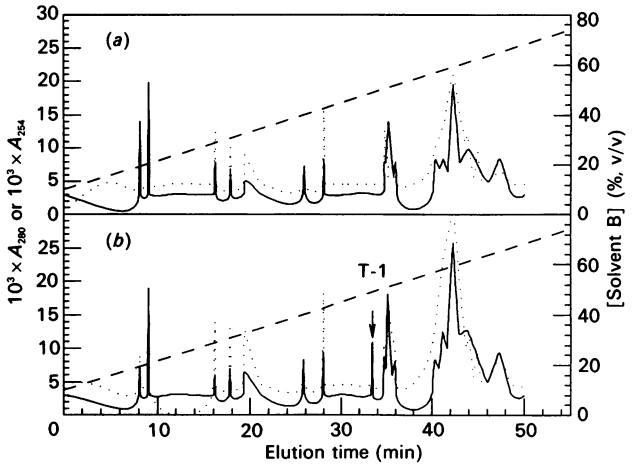

Fig. 4. H.p.l.c. pattern of the tryptic digest of Nbs-modified streptavidin

Nbs-modified streptavidin–biotin complex (*a*) and Nbs-modified streptavidin (*b*) were treated for 20 h with trypsin as detailed in the text. The digests were chromatographed on a Lichrosphere Select B RP-8 column, with a linear gradient of solvent B containing 50% propanol-1-ol in 0.1% trifluoroacetic acid (——) as described in the text. The eluted peptides were monitored by absorbance at 280 nm (·····) and at 254 nm (——). A single additional peak (T-1) appeared in panel (*b*), presumed to contain modified tyrosine, located in the biotin-binding site.

subunit can be rapidly modified upon addition of a large excess of Nbs-F. As above, the correlation of the modified tyrosine fraction with biotin-binding activity was determined in accordance with Tsou (1962) (Fig. 3). Statistical analysis of the data indicated that modification of an average of one tyrosine residue (out of the three modified per streptavidin subunit) is sufficient to inactivate completely the biotin-binding activity of the protein. Moreover, the streptavidin–biotin complex is less reactive with the reagent; only two tyrosine residues per subunit are modified under conditions similar to those applied to the free protein, indicating that, on the average, one of the tyrosine residues is protected biotin (Fig. 3 inset).

The identification of the essential tyrosine residue(s) and its location in the primary sequence was performed in the following manner. Two identical samples of streptavidin were prepared. One was preincubated with an excess of biotin, and both samples were treated with the same amount of the reagent. The dialysed and freeze-dried Nbs-modified samples were then dissolved in 10 M-urea, boiled and dialysed successively against 2 M-urea (to eliminate biotin from the solution) and water, and the contents of the dialysis bag were subjected to tryptic hydrolysis. The resultant peptides were fractionated by reverse-phase h.p.l.c. (Fig. 4). The enhanced absorbance at 254 nm compared with that at 280 nm indicated the presence of Nbs-modified tyrosine in the given peptide. Fig. 4(*a*) shows the h.p.l.c. profile of the sample preincubated with biotin, and several Nbs-modified tyrosine fractions appear in the chromatogram. Fig. 4(*b*) shows the h.p.l.c. profile of the trypsin-hydrolysed Nbs-modified streptavidin without biotin. All peptide fractions appear identical in the two profiles, with the exception of a single additional peak with high absorbance at 254 nm in Fig. 4(*b*) (indicated by the arrow). This fraction is assumed to include the peptide containing the tyrosine residue of interest, i.e. the tyrosine residue positioned in the biotin-binding site that is shielded after biotin binding. This peptide (designated T-1), which contains on average about 0.2 modified tyrosine residue per subunit, was therefore isolated and subjected to gas-phase sequence analysis (Table 1). The

Table 1. *N*-Terminal sequence determination of Nbs-modified tyrosine-containing streptavidin peptides

Peak T-1 is the tryptic fraction denoted in Fig. 4, and peaks C-1 and C-2 are the designated chymotryptic–tryptic fractions in Fig. 5. Xaa signifies an unknown amino acid peak, presumed to represent Nbs-modified Tyr.

N-Terminal cycle	Peak T-1	Peak C-1	Peak C-2
1	Xaa	Ile	Ile
2	Val	Val	Val
3	Leu	Thr, Leu	Thr
4	Thr	Ala, Thr	Ala
5	Gly	Gly	Gly
6		Ala	Ala
7		Asp*	Asp*
8			Gly
9			Ala
10			Leu
11			Thr
12			Gly
13			Thr
14			Xaa
15			Glu

* Repetitive yields of Edman degradation generally ranged between 30% and 70%, with the exception of that of the Asp–Gly bond, the yield of which was much lower. Aspartic acid tends to undergo cyclization to the iminosuccinyl derivative. The reaction is accelerated by the glycine residue that follows in the primary sequence (Bodansky *et al.*, 1967). In the case of peak C-1, most of the material underwent cyclization and very little remained for further sequence analysis. In the case of peak C-2, the large quantity of sample derived from h.p.l.c. (see Fig. 5) enabled the detection of 15 sequence cycles even after extensive cyclization of the Asp–Gly bond.

isolated peptide was found to consist of amino acid residues 54–58, and include a single tyrosine residue (Tyr-54).

Owing to the relatively low yield of peptide T-1 and taking into consideration the sequence similarity between the two tyrosine-containing stretches in avidin and streptavidin, Tyr-54 was apparently not the major tyrosine residue that we had originally

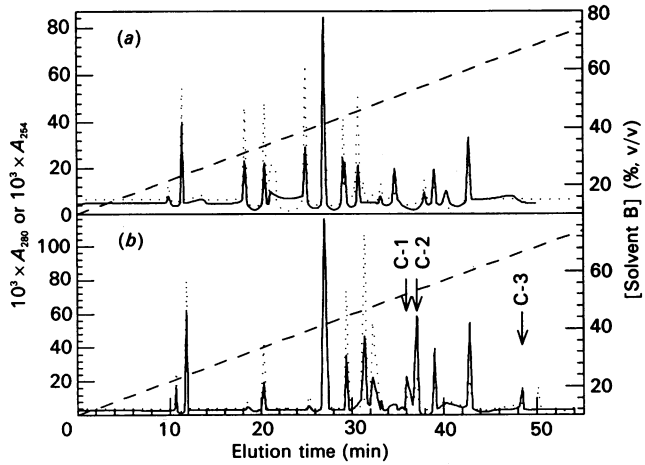

Fig. 5. H.p.l.c. pattern of the chymotryptic–tryptic digest of Nbs-modified streptavidin

Nbs-modified streptavidin–biotin complex (*a*) and Nbs-modified streptavidin (*b*) were treated successively for 20 h with trypsin and for 2 h with chymotrypsin. The resultant peptides were separated as detailed for Fig. 4. Three additional fractions containing Nbs-modified tyrosine were detected in panel (*b*) and were designated C-1, C-2 and C-3.

suspected would be modified with the reagent, and hence that would be directly involved in the biotin-binding process. Tyr-43 of streptavidin (the analogue of Tyr-33 of egg-white avidin) is located in a relatively large (46-amino-acid-residue) tryptic peptide (residues 8–53) that includes another tyrosine residue (Tyr-22). In this case a single modification may not significantly affect the elution pattern of such a large peptide; were this the case, we would not detect the difference in the complex pattern shown in Fig. 3. Therefore the two modified streptavidin samples were further subjected to chymotryptic hydrolysis. The resultant peptides were separated by reverse-phase h.p.l.c. (Fig. 5). Three additional peaks containing Nbs-modified tyrosine could be detected (Fig. 5*b*) in the digest of the unprotected form of the modified protein. These three fractions were pooled separately and subjected to *N*-terminal sequence analysis (Table 1). Fraction C-1 (consisting of about 25% of the differentially modified peptides) was found to contain a mixture of two peptides; one is the previously isolated and characterized peptide T-1 (containing the Nbs-modified Tyr-54) and the second is the 14-amino-acid-residue peptide (residues 30–43) that contains unmodified Tyr-43. Fraction C-2 (about 70% of the differentially modified peptides) represents the 24-amino-acid-residue peptide (residues 30–53) that includes modified Tyr-43. The third fraction (C-3) did not contain detectable quantities of amino acid and probably represents a degradation product(s) of the reagent. It is to be noted that chymotrypsin was not very efficient in hydrolysing near the Nbs-modified tyrosine residues.

DISCUSSION

The question that we asked ourselves in the study of these two proteins concerned the possible role of the single tyrosine (Tyr-33) in avidin and its analogue (Tyr-43) in streptavidin. Previously we had tried to answer this question by using photochemically induced dynamic nuclear polarization (Gitlin *et al.*, 1989). However, using this technique we were not able to establish the role of the tyrosine residues in the biotin-binding site, since the lone tyrosine residue of the avidin subunit is buried both in the presence and in the absence of biotin and is not available for interaction with the dye. Presumably the Tyr-43 analogue is also buried in the streptavidin subunit.

From the evidence furnished by this study, it seems that the single tyrosine residue in the avidin subunit indeed plays an important role in the biotin binding. In this regard, one of the interesting and surprising findings was that avidin modified by Nbs-F requires an average of only 0.5 mol of reagent/mol of subunit to be fully inactivated. These results can be interpreted such that the modification of a tyrosine residue in one binding site of the tetramer will cause a critical alteration in the binding site of an adjacent subunit. The avidin–biotin complex does not react with this reagent, indicating further that the tyrosine residue is located inside the biotin-binding cleft.

The minor extents of modification of Tyr-54 and its partial protection by biotin were, perhaps, unexpected; however, it may be noted that this residue occurs at a position similar to that of Lys-45 in avidin, which was proposed to be near the biotin-binding site on the basis of its reactivity with 2,4-dinitrophenyl fluoride. Alternatively, these residues may be exposed in the absence of biotin but buried consequent to formation of the avidin–biotin complex.

The finding that Tyr-43 of streptavidin is both labelled by Nbs-F and protected by biotin confirms our initial supposition concerning the contribution of this amino acid residue to the biotin-binding site in avidin. In retrospect, the conservation of the two analogous tyrosine residues of avidin and streptavidin (Tyr-33 and Tyr-43 respectively) in the longest single homologous stretch in the primary sequences of the two proteins would in itself tend to implicate their salience to the corresponding binding site. These results also agree with the recent X-ray structure of streptavidin (Weber *et al.*, 1989), which demonstrates a critical interaction between Tyr-43 with the ureido ring of biotin. The prospective solution of the three-dimensional structure of avidin (O. Livnah, M. Wilchek, Y. Hiller, E. A. Bayer & J. Sussman, unpublished work; see Wilchek & Bayer, 1989) will undoubtedly clarify the extent of structural similarity between the two biotin-binding proteins.

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