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Studies on the biotin-binding site of streptavidin

Tryptophan residues involved in the active site

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Streptavidin, the non-glycosylated bacterial analogue of the egg-white glycoprotein avidin, was modified with the tryptophan-specific reagent 2-hydroxy-5-nitrobenzyl (Hnb) bromide. As with avidin, complete loss of biotin-binding activity was achieved upon modification of an average of one tryptophan residue per streptavidin subunit. Tryptic peptides obtained from an Hnb-modified streptavidin preparation were fractionated by reversed-phase h.p.l.c., and three major Hnb-containing peptide fractions were isolated. Amino acid and *N*-terminal sequence analysis revealed that tryptophan residues 92, 108 and 120 are modified and probably comprise part of the biotin-binding site of the streptavidin molecule. Unlike avidin, the modification of lysine residues in streptavidin failed to result in complete loss of biotin-binding activity. The data imply subtle differences in the fine structure of the respective biotin-binding sites of the two proteins.

INTRODUCTION

Interest in the avidin–biotin complex has persisted over the past several decades for a combination of reasons. On the one hand, the extraordinary affinity constant (K_a approx. 10^{15} M^{-1}) that exists between the egg-white glycoprotein avidin and the vitamin biotin has attracted a considerable amount of scientific activity designed to understand better the molecular forces behind this remarkable interaction (Green, 1975). On the other hand, the unique features of this system have enabled the widespread practical application of the avidin–biotin complex (Bayer & Wilchek, 1980; Wilchek & Bayer, 1984, 1988).

Perhaps no less remarkable is the discovery more than two decades ago (Stapley *et al.*, 1963; Chaiet & Wolf, 1964) of a second, evolutionarily unrelated, biotin-binding protein from the bacterium *Streptomyces avidinii*. Interestingly, the bacterial protein exhibits both physical and affinity properties very similar to those of the egg-white protein. Thus both streptavidin and avidin are tetramers composed of homologous subunits, each of which bears a single biotin-binding active site. Some differences characterize the two molecules; whereas avidin is a basic glycoprotein, streptavidin is a neutral non-glycosylated protein.

Although recent evidence (Bayer *et al.*, 1986; Argarana *et al.*, 1986) demonstrates that the true molecular mass of streptavidin is significantly higher than that of avidin, the bacterial protein commonly occurs in a stable truncated form, termed 'core' streptavidin (Pähler *et al.*, 1987), the size of which is almost identical with that of the egg-white protein. More significantly, a relatively high degree of homology characterizes the sequences of streptavidin and avidin (Argarana *et al.*, 1986). Notably, two separate Trp-Lys sequences are conserved in the two proteins, thereby underscoring previous suggestions that both tryptophan and lysine residues may be involved in biotin binding (Green, 1975).

In this context, we have recently reported that avidin loses its biotin-binding activity after modification of lysine residues by 1-fluoro-2,4-dinitrobenzene (Gitlin *et al.*, 1987) or by treatment with the tryptophan-specific reagent 2-hydroxy-5-nitrobenzyl (Hnb) bromide (Gitlin *et al.*, 1988). The results indicated that modification of any of three individual lysine residues (Lys-45, Lys-94 or Lys-111) or modification of either of two tryptophan residues (Trp-70 or Trp-110) leads to the complete loss of biotin-binding activity.

In view of the striking structural and functional similarities between avidin and streptavidin, the obvious question is whether homologous amino acid moieties in streptavidin are susceptible to similar group-specific modifications. The present paper deals with the results of an analogous approach performed on streptavidin. The data indicate that tryptophan behaves similarly in the two proteins, but no conclusion could be drawn concerning the involvement of lysine residues in the biotin-binding site of streptavidin.

MATERIALS AND METHODS

Materials

2-Hydroxy-5-nitrobenzyl bromide (Koshland I reagent), biotin and trypsin [L-1-chloro-4-phenyl-3-tosylamidobutan-2-one ('TPCK')-treated] were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 1-Fluoro-2,4-dinitrobenzene was obtained from Fluka A.G. (Buchs, Switzerland). Streptavidin was isolated from the broth of *Streptomyces avidinii*, and the native (untruncated) form was purified by the procedure of Bayer *et al.* (1986) with the use of an iminobiotin–Sepharose column.

Biotin-binding assay for avidin activity

The assay was based on the capacity of known concentrations of native streptavidin or Hnb-modified

Abbreviations used: Hnb, 2-hydroxy-5-nitrobenzyl; Dnp, 2,4-dinitrophenyl.

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streptavidin to bridge between biotinyl-albumin-coated micro-titre plates and biotinylated alkaline phosphatase (Bayer *et al.*, 1985).

Lysine modification

Samples (0.5 ml) containing streptavidin (0.4 mg/ml) were treated with 1-fluoro-2,4-dinitrobenzene in 0.1 M-$NaHCO_3$, pH 9.0. The ratio of reagent ranged from 0.4:1 to 10:1 with respect to the molar concentration of streptavidin subunit. The reaction was carried out for 1 h at 40 °C, after which the solution was dialysed against distilled water.

Tryptophan modification

Samples (1.0 ml) containing streptavidin (0.5 mg of protein/ml) were treated with Hnb-Br at pH 2.7. The ratio of reagent ranged from 0.4:1 to 10.0:1 with respect to the molar concentration of streptavidin subunit. The reaction was carried out for 5 min at room temperature with vigorous magnetic stirring. The solution was then dialysed against water.

In each case the extent of Hnb modification was determined spectrophotometrically [$\epsilon_{410} = 18400\ M^{-1} \cdot cm^{-1}$ (pH 11)], and the final concentration of protein was determined by the method of Malin *et al.* (1985).

For preparative work, a sample of streptavidin (6.2 mg in 1 ml) was treated under the above conditions with Hnb-Br (0.96 mg dissolved in 30 μl of dry acetone).

Tryptic hydrolysis

A dialysed sample (6.2 mg in 1 ml) of the Hnb-modified protein was boiled for 10 min and treated with trypsin (0.12 mg) for 20 h at 40 °C with addition of 0.2 ml of 0.1 M-$NaHCO_3$, pH 8.0. A solution (15 μl) of 10% (v/v) trifluoroacetic acid was added to the reaction mixture, and the protein precipitate was centrifuged. A 0.1 ml portion of the supernatant was subjected to reversed-phase h.p.l.c. under the following conditions: a Lichrosphere Select B RP-8 (Merck) 250 mm × 4 mm cartridge column was used for peptide separation; a 90 min linear gradient was initiated with 100% of solvent A [0.1% (v/v) trifluoroacetic acid in water] ending with 90% of solvent B [0.1% (v/v) trifluoroacetic acid in 50% (v/v) propan-2-ol]. The flow rate was calibrated at 0.4 ml/min. The corresponding fractions from the Hnb-containing peaks were collected. The respective amino acid content was established by amino acid analysis, and the respective *N*-terminal sequence was determined by use of a gas-phase sequencer.

RESULTS

Treatment of streptavidin with Hnb-Br led to a loss of biotin-binding activity. Upon varying the quantity of Hnb incorporated into the protein, the extent of inhibition in biotin binding was proportionate to the amount of modified residues (Fig. 1). Modification of only one tryptophan residue caused the complete loss of binding. When streptavidin was preincubated with biotin, Hnb failed to label the protein (Fig. 1 inset), reflecting a protection of the tryptophan residues in the binding site.

In contrast with the results with Hnb, only partial inactivation of biotin binding could be achieved by modifying the lysine residues in streptavidin with 1-fluoro-2,4-dinitrobenzene (Fig. 2). Moreover, upon

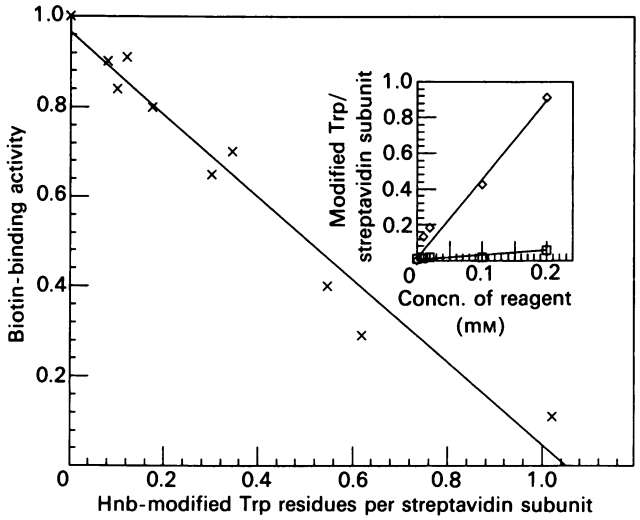

Fig. 1. Effect of tryptophan modification on the biotin-binding activity of streptavidin

The fraction of activity remaining after modification (compared with the activity of the native protein) is plotted against the number of modified tryptophan residues. Modification of an average of one residue per subunit resulted in 100% loss of activity. Inset: protection of biotin-binding site from Hnb modification. Solutions (28 nmol of streptavidin subunit in 1 ml) containing either free streptavidin (◇) or the streptavidin–biotin complex (□) were treated with the indicated final concentrations of Hnb-Br.

preincubation of streptavidin with biotin, only partial protection of the lysine residues from modification was obtained (Fig. 2 inset).

In order to determine which tryptophan residue(s) is (are) modified in streptavidin, a sample of streptavidin containing 1.16 Hnb residues per subunit was subjected to hydrolysis with trypsin, and the tryptic peptides were separated by reversed-phase h.p.l.c. The elution profile is illustrated in Fig. 3. Three major Hnb-containing peaks were observed and termed T-1, T-2 and T-3 according to their order of elution. Five or six minor peaks were also observed, which collectively accounted for less than 20% of the total label; these minor peaks were not investigated further in this study.

Column fractions comprising each of the major Hnb-labelled peptides were pooled, and their respective amino acid contents were determined (Table 1). Analysis of peptide T-1 coincided with that of the tryptic peptide containing residues 85–103. One tryptophan residue, Trp-92, is contained in this peptide. Amino acid analysis of peptides T-2 and T-3 revealed that both peaks represented the same peptide (residues 104–121). Two tryptophan residues are contained in this peptide, and the resolution of the labelled peptide by h.p.l.c. into two distinct peaks, each of which contains a single labelled residue, indicates that a different tryptophan is labelled in each case. It remained to be decided which position was labelled in each of the isolated peptides. This was accomplished by *N*-terminal sequence determination (Table 2), the results of which demonstrated that peptide T-2 is the peptide that bears a tryptophan residue labelled in position 120 and that peptide T-3 contains Hnb-labelled Trp-108.

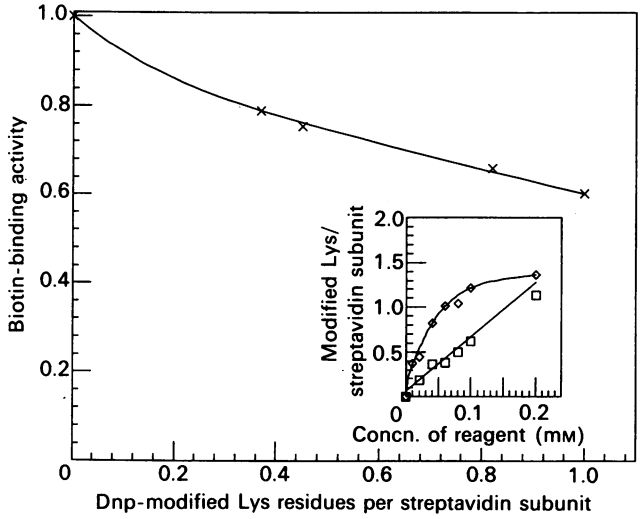

Fig. 2. Effect of lysine modification on the biotin-binding activity of streptavidin

The percentage of biotin-binding activity remaining after 1-fluoro-2,4-dinitrobenzene modification (compared with the activity of the native protein) is plotted against the number of modified lysine residues. Inset: attempts to protect lysine residues from undergoing Dnp modification. Solutions (11 nmol of streptavidin subunit in 0.5 ml) containing either free streptavidin (◇) or the streptavidin–biotin complex (□) were treated with the indicated final concentrations of 1-fluoro-2,4-dinitrobenzene.

Table 1. Amino acid composition of streptavidin peptides containing Hnb-Trp

The values were normalized to alanine. The values in parentheses represent the actual number of amino acid residues in the Hnb-Trp-containing peptides of streptavidin. In peptides containing both unmodified and Hnb-modified tryptophan, the relative amounts of each were determined spectroscopically assuming a value of 1 residue per peptide for Hnb-Trp.

	Amino acid composition (residues/molecule of peptide)		
Amino acid	Peptide T-1 (residues 85–103)	Peptide T-2 (residues 104–121)	Peptide T-3 (residues 104–121)
Asx	1.5 (1)	2.0 (2)	2.0 (2)
Thr	1.7 (2)	3.2 (4)	2.4 (4)
Ser	1.8 (2)	1.1 (1)	1.3 (1)
Glx	2.3 (2)	1.7 (2)	1.6 (2)
Gly	3.4 (3)	1.3 (1)	1.7 (1)
Ala	4.0 (4)	2.0 (2)	2.0 (2)
Val	1.2 (1)		
Ile		0.6 (1)	0.6 (1)
Leu		1.6 (2)	1.7 (2)
Tyr	1.1 (1)		
His	0.9 (1)		
Lys		1.2 (1)	1.1 (1)
Arg	0.9 (1)		
Trp		1.2 (2)	0.8 (2)
Hnb-Trp	1.0 (1)	1.0	1.0

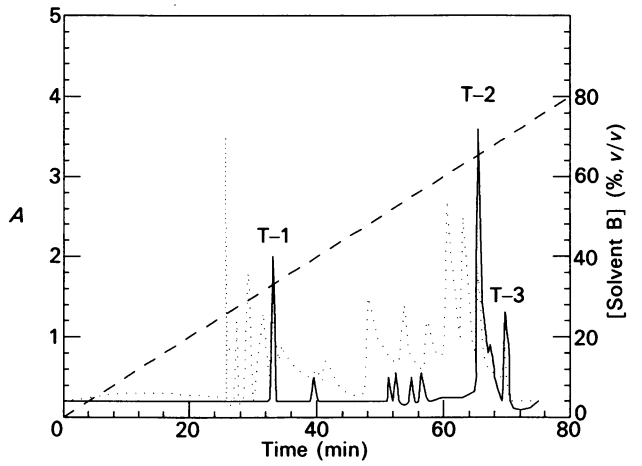

Fig. 3. H.p.l.c. pattern of the tryptic digest of Hnb-modified streptavidin

Hnb-modified streptavidin was treated for 20 h with trypsin as detailed in the text. The digest was chromatographed on a Lichrosphere Select B RP-8 column with a linear gradient of solvent B (----) as described in the text. The eluted peptides were monitored by absorbance at 220 nm (·····) and 320 nm (——). The positions of the major Hnb-modified peptides (T-1, T-2 and T-3) are indicated.

Table 2. *N*-Terminal sequence determination of Hnb-Trp-containing streptavidin peptides

X signifies an unknown peak, presumed to represent Hnb-modified tryptophan, which appeared at an intermediate position between the diphenylthiourea and tryptophan peaks.

N-Terminal cycle	Peptide T-1	Peptide T-2	Peptide T-3
1	Asn	Ile	Ile
2	Ala	Asn	Asn
3	His	Thr	Thr
4	Ser	Gln	Gln
5	Ala	Trp	X
6	Thr	Leu	Leu
7	Thr	Leu	Leu

Trp-120 contains most of the label (62%, corrected for the three residues). Of the remainder, Trp-92 contains 21% and Trp-108 contains 17% of the label.

DISCUSSION

In view of the reported similarities in the biological properties and physical structure of avidin and streptavidin, it was decided to determine whether the two biotin-binding proteins are also related chemically, placing particular emphasis on the comparative structure of the respective binding sites.

In this regard, we have previously shown that at least two tryptophan residues per subunit of avidin are involved in the binding of biotin by using the tryptophan-specific reagent Hnb-Br. We have also shown that

modification of an average of one lysine residue per avidin subunit with 1-fluoro-2,4-dinitrobenzene also inhibits the binding of biotin. We therefore performed a cognate study on streptavidin, using the same reagents. The results demonstrate clearly that tryptophan is also involved in biotin binding to streptavidin, since introduction of one residue of Hnb results in a concomitant loss of biotin binding. At high concentrations of Hnb-Br, several tryptophan residues are modified. If, however, the reaction is terminated after an average of one tryptophan residue is modified, virtually no biotin-binding activity is detected. Under these conditions three tryptophan residues (Trp-92, Trp-108 and Trp-120) are modified in different proportions, suggesting that these three residues are involved in the binding of biotin to streptavidin, perhaps by forming some kind of cleft or pocket built mainly of tryptophan residues.

It is also noteworthy that, of the two tryptophan residues 'conserved' in the Trp-Lys sequences in both avidin and streptavidin, one of these (Trp-120) accounted for most (> 60%) of the Hnb label whereas the other (Trp-79) appeared to be virtually unlabelled. It should be remembered that the analogous tryptophan residues in avidin (70 and 110) were both labelled about equally. This does not necessarily mean that in streptavidin Trp-79 is not part of the active site. It is equally conceivable that this residue occupies a more buried position within the active site and that the other tryptophan residues (Trp-92, Trp-108 and particularly Trp-120) interact with the reagent before it can reach Trp-79.

In such studies it is common to find examples in which only one specific residue in a protein is modified and that modification of this residue leads to the complete loss of activity. When more than a single residue is modified, such a modification provides much more information concerning the residues in the binding site. In the case of streptavidin, three tryptophan residues are modified by Hnb-Br, suggesting that all three residues may be involved in the active site. It thus seems that the electronic structure of the indole group provides an excellent source for forming strong binding sites, resulting in an efficient interaction even with such a simple molecule as biotin. The affinity of this interaction appears to be proportional to the number of trytophan residues in the site. This finding has prompted a recent study in our laboratory to determine whether artificial biotin-binding sites can be constructed by using co-polymers of tryptophan with other amino acids (G. Gitlin, E. A. Bayer & M. Wilchek, unpublished work).

In contrast with tryptophan residues, the involvement of lysine in streptavidin is more difficult to assess, since only a partial loss of binding activity could be demonstrated upon modification of streptavidin with 1-fluoro-2,4-dinitrobenzene. The question is, therefore, whether lysine is involved at all in the binding of biotin to avidin or streptavidin. In our previous study on the lysine residues of avidin (Gitlin *et al.*, 1987), we suggested either that lysine residues may indeed be located in the binding site or that the Dnp label may block other functional groups (i.e. tryptophan residues) that actively interact with biotin in the binding site of avidin. In the latter case Dnp-Lys may block the tryptophan group(s) by forming a charge-transfer complex with the indole ring, thus preventing biotin from binding; lysine by itself need not be involved in the binding process as such. In this context we have recently shown that aromatic nitro compounds have an affinity for avidin (G. Gitlin, E. A. Bayer & M. Wilchek, unpublished work). Such a binding of aromatic compounds should not have such a strong inhibiting effect upon biotin binding, since the dye 2-(4'-hydroxyazobenzene)benzoic acid (which binds to the biotin-binding site of avidin with a relatively low affinity constant of 10^6 M^{-1}) is displaced very easily by biotin (Green, 1975). Such a strong effect of the Dnp group may be due to the first-order interaction (emanating from within the active site) between the indole ring and the Dnp group, as opposed to a second-order interaction (albeit very strong) between biotin and avidin.

The differences observed in the present study concerning the reactivities of tryptophan and lysine residues in avidin and streptavidin apparently reflect subtle structural differences in the biotin-binding sites of the two proteins. Such differences could account for the previously reported differential recognition characteristics for biotin, e.g. in avidin only the ureido ring of the vitamin is necessary for strong binding whereas in streptavidin the integrity of the bicyclic ring system is required. Further information concerning the exact contribution of individual tryptophan and lysine residues will be clarified only after X-ray analysis of avidin and streptavidin.

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