Biochem. J. (1988) 250, 291–294 (Printed in Great Britain)

Studies on the biotin-binding site of avidin

Tryptophan residues involved in the active site

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Egg-white avidin was modified with the tryptophan-specific reagent 2-hydroxy-5-nitrobenzyl bromide. The complete loss of biotin-binding activity was achieved upon modification of an average of one tryptophan residue per avidin subunit. The identity of the modified residues was determined by isolating the relevant tryptic and chymotryptic peptides from CNBr-cleaved avidin fragments. The results demonstrate that Trp-70 and Trp-110 are modified in approximately equivalent proportions. It is believed that these residues are located in the active site of avidin and take part in the binding of biotin.

INTRODUCTION

Interest in the egg-white protein avidin has increased in recent years, owing to its extensive use as a universal tool in the biochemical sciences (Bayer & Wilchek, 1980; Wilchek & Bayer, 1984). Avidin has been shown to be a basic glycoprotein containing four subunits having a combined molecular mass of 67000 Da (Green, 1975). The primary structure of avidin contains 128 amino acid residues and one biotin-binding site per subunit (DeLange & Huang, 1971). The avidin–biotin complex represents the strongest biochemical (non-covalent) interaction known in Nature between a ligand and a protein. The reason for this strong interaction is not entirely understood, even after decades of work since avidin was first isolated (Eakin *et al.*, 1941) and the uniqueness of its biotin-binding properties established.

Although the biotin-binding activity of avidin was relatively insensitive to a variety of group-specific chemical reagents (Fraenkel-Conrat *et al.*, 1952), both tryptophan and lysine residues have been implicated in the active site. We (Gitlin *et al.*, 1987) have recently extended Green's (1963, 1975) earlier studies on the involvement of lysine, and have shown that lysine residues 45, 94 and 111 are modified by 1-fluoro-2,4-dinitrobenzene and probably comprise part of the biotin-binding site.

Initial evidence that directly implicated tryptophan as an essential amino acid in the avidin active site emanated from biotin-induced difference spectra (Green, 1963). These studies were supported by the sensitivity of avidin to oxidation by *N*-bromosuccinimide (Green, 1962, 1975). With this reagent all four tryptophan residues of each avidin subunit were rapidly oxidized at pH 4.6, and biotin-binding activity was lost when an average of two had been destroyed. In subsequent experiments Huang (1971) showed that when half the activity was lost about half of Trp-10 and Trp-70 were destroyed. The evidence on Trp-110 was inconclusive.

Although it is likely that several tryptophan residues contribute to biotin binding, the precise location of the reactive tryptophan residue(s) in the primary sequence has not been unequivocally elucidated. All the methods described above used spectroscopic techniques (sometimes in conjunction with chemical modification) to predict the involvement of tryptophan in the biotin-binding site of avidin. With such techniques, however, it is difficult to locate precisely which tryptophan in the primary structure is involved. We therefore decided to use 2-hydroxy-5-nitrobenzyl bromide (Hnb-Br) for this purpose (Horton & Koshland, 1965). This reagent is coloured and very selective for tryptophan, thereby allowing the localization of the modified residue(s). We could thus use the reagent as a tool for isolating the peptide(s) involved.

Our results showed that the modification of only one tryptophan residue per subunit is sufficient to destroy biotin binding completely. Moreover, this could be accomplished by the modification of either of two individual residues, namely Trp-70 or Trp-110.

MATERIALS AND METHODS

Materials

Avidin was generously provided by Soc. Coop. Belovo (Bastogne, Belgium). Hnb-Br (Koshland I reagent), biotin, chymotrypsin 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.). CNBr was obtained from Fluka A.G. (Buchs, Switzerland).

Biotin-binding assay for avidin activity

The assay was carried out essentially as described previously (Bayer *et al.*, 1985). Samples (0.1 ml) containing known concentrations of avidin or Hnb-modified avidin were applied to the wells of micro-titre plates coated with biotinyl-(bovine serum albumin), and subsequent interaction with biotinyl-(alkaline phosphatase) was measured.

Tryptophan modification

The reaction was performed in 0.5 ml samples (2 mg of protein/ml) by using Hnb-Br at pH 2.7 with ratios of reagent ranging from 0.4 to 10.0 with respect to the molar

Abbreviation used: Hnb, 2-hydroxy-5-nitrobenzyl.

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concentration of avidin 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 as described by Malin *et al.* (1985).

For preparative work, a sample of avidin (32 mg in 5 ml) was treated under the above conditions with Hnb-Br (2.3 mg dissolved in 0.2 ml of dry acetone).

CNBr cleavage

The Hnb-modified avidin sample was treated with CNBr (80 mol/mol of methionine) in 70% (v/v) formic acid for 20 h at 25 °C by the method of Gross & Witkop (1962) as modified by Steers *et al.* (1965). The reaction mixture was evaporated to dryness. The residue was dissolved in 99% (v/v) formic acid, diluted to 50% formic acid and applied to a Sephadex G-50 column (2 cm × 100 cm), pre-equilibrated and eluted with 30% (v/v) acetic acid. The eluted peptides were pooled and the fractions were freeze-dried. Samples were adjusted to pH 11 by addition of NaOH, and the extent of modification per fraction was determined.

Chymotryptic hydrolysis

The desired CNBr-cleaved Hnb-containing peptide fraction was hydrolysed by chymotrypsin (0.2 mg) for 2 h at 40 °C in 2 ml of 0.05 M-$NaHCO_3$, pH 8.0. The reaction mixture was freeze-dried and dissolved in 50% formic acid. The solution was subjected to gel filtration on a Sephadex G-25 column (1.5 cm × 100 cm), pre-equilibrated and eluted with 30% acetic acid.

Tryptic hydrolysis

The second CNBr-cleaved Hnb-containing peptide fraction was hydrolysed further by TPCK-treated trypsin (0.2 mg) for 20 h at 40 °C in 2 ml of 0.05 M-$NaHCO_3$, pH 8.0. The reaction mixture was freeze-dried and dissolved in 50% formic acid. The solution was applied to a Sephadex G-25 column as described above. The respective fractions from each Hnb-containing peak were pooled, and the corresponding amino acid content was determined by amino acid analysis (D550 analyser; Durrum Instrumental Corp., Palo Alto, CA, U.S.A.).

RESULTS

In order to determine the relationship between biotin-binding activity and the extent of tryptophan modification, avidin was treated with various amounts of Hnb-Br, and, correspondingly, different quantities of Hnb were incorporated into the protein. The absorption spectrum of avidin containing 0.96 Hnb group per subunit is depicted in Fig. 1. The biotin-binding activity of avidin decreased as the amount of modified tryptophan increased. By plotting the results, the contribution of the modification to inactivation of avidin could be determined (Fig. 2). The results show that modification of only one tryptophan residue per subunit is sufficient to cause complete loss of biotin binding. The observed inactivation could be prevented by biotin (Fig. 2 insert). The fact that the avidin–biotin complex was not modified by this reagent suggests that tryptophan(s) is a part of the binding site.

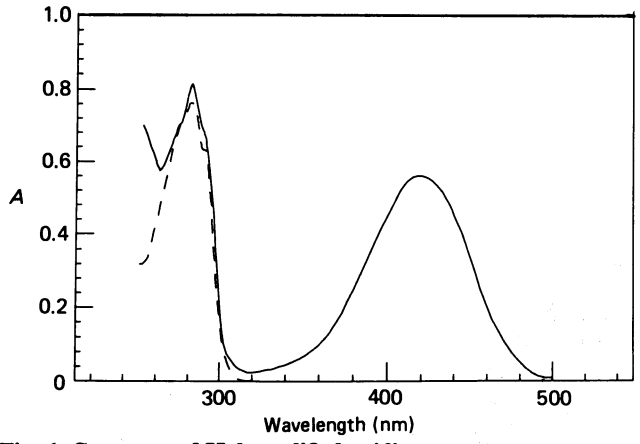

Fig. 1. Spectrum of Hnb-modified avidin

Solution (0.55 mg/ml) of Hnb-modified (——) or native (– – – –) avidin were examined in a Cary model 14 spectrophotometer.

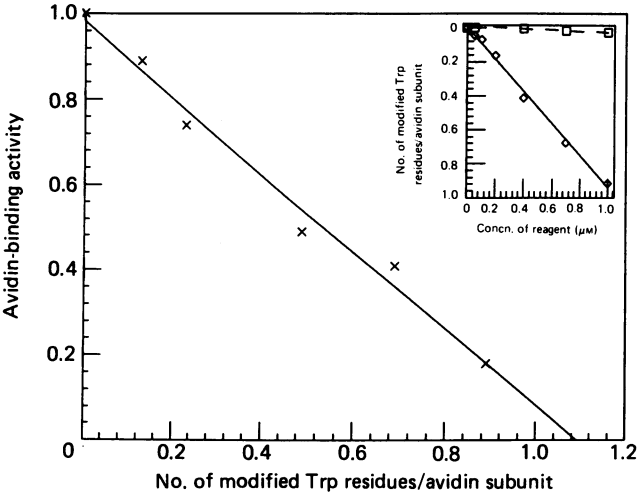

Fig. 2. Effect of the modification of tryptophan residues on the binding of biotin by avidin

The fraction of the activity remaining after modification (compared with the activity of the native protein) is plotted against the number of modified tryptophan residues. Modification of one residue per subunit resulted in 100% loss of activity. Inset: protection of biotin-binding site from Hnb modification. Solutions (1 ml, 0.1 μM in avidin subunits), containing either avidin (◇) or the avidin–biotin complex (□), were treated with different final concentrations (ranging from 0.05 to 1.0 μM) of Hnb-Br.

A sample of avidin containing 0.96 Hnb residue per subunit was subjected to CNBr cleavage, and the resultant peptides were separated by gel filtration on Sephadex G-50. The elution profile is illustrated in Fig. 3. On the basis of amino acid analysis of the isolated fractions, fraction B corresponded to the 32-residue *C*-terminal peptide. Fraction A-1 contained native (uncleaved) protein, and fraction A-2 was determined to be the CNBr-cleaved fragment that lacks the 32-residue *C*-terminal peptide. Hnb-containing fractions A-1 and A-2 were each passed again through the Sephadex G-50 column to remove cross-contaminating material.

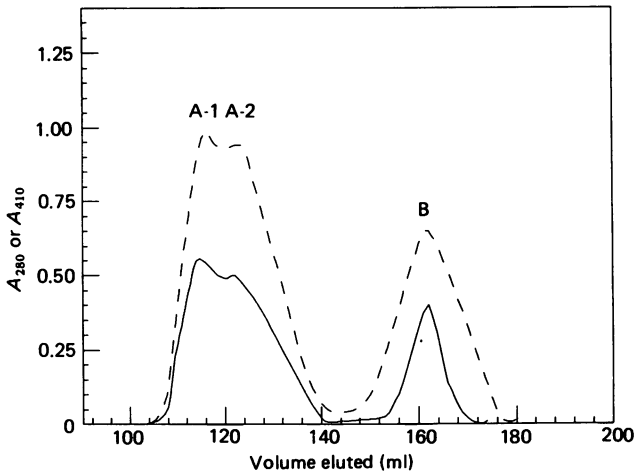

Fig. 3. Gel-filtration pattern of CNBr-cleaved Hnb-modified avidin

Hnb-modified avidin was treated for 20 h with CNBr as detailed in the text. The product was chromatographed on Sephadex G-50, and the eluted peptides were monitored by their A_{280} (-----) and A_{410} (——).

Table 1. Amino acid composition of avidin peptides containing Hnb-tryptophan

The values were normalized to those of valine. The values in parentheses represent the actual number of amino acids in the Hnb-tryptophan-containing peptides of avidin. The extent of Hnb-Trp formation was determined spectroscopically.

	Amino acid composition (residues/molecule of peptide)	
Amino acid	Peak A-3 (residues 67–71)	Peak B-1 (residues 101–111)
Asx	0.9 (1)	4.2 (4)
Thr	0.6 (1)	
Ser		1.7 (2)
Gly		1.3 (1)
Val	1.0 (1)	1.0 (1)
Ile		0.9 (1)
Lys	1.1 (1)	1.1 (1)
Hnb-Trp	1.0	1.0

Fraction A-2 was further hydrolysed by chymotryptic cleavage, in order to determine whether only one or both tryptophan residues contained in this segment are labelled. The digest was separated by column chromatography on Sephadex G-25. Only one fraction (A-3 in Fig. 4) contained the Hnb moiety. The amino acid content of this fraction was determined. As shown in Table 1, the modified tryptophan residue in fraction A-3, which contained about 50% of the incorporated Hnb, corresponded to Trp-70.

Fraction B (Fig. 3) also contained two tryptophan residues (97 and 110). In order to determine which

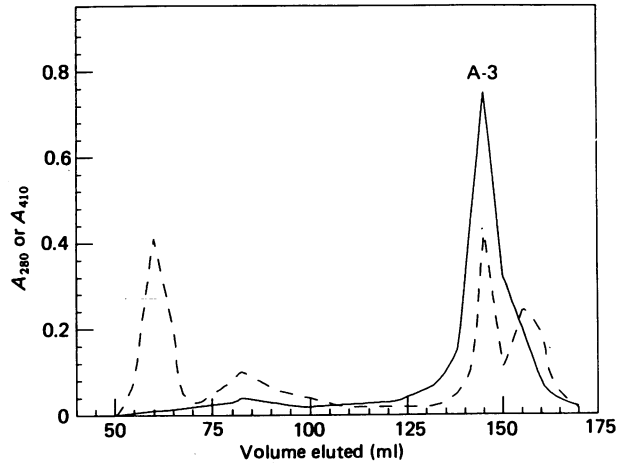

Fig. 4. Gel-filtration pattern showing the chymotryptic digest of the large CNBr-cleaved fragment of Hnb-modified avidin

Fraction A-2 (see Fig. 3) of the Sephadex-G-50-chromatographed CNBr-cleaved Hnb-modified avidin was digested with chymotrypsin for 2 h as detailed in the text. The digest was chromatographed on a Sephadex G-25 column. Peptide elution was monitored by the A_{280} (-----) and A_{410} (——).

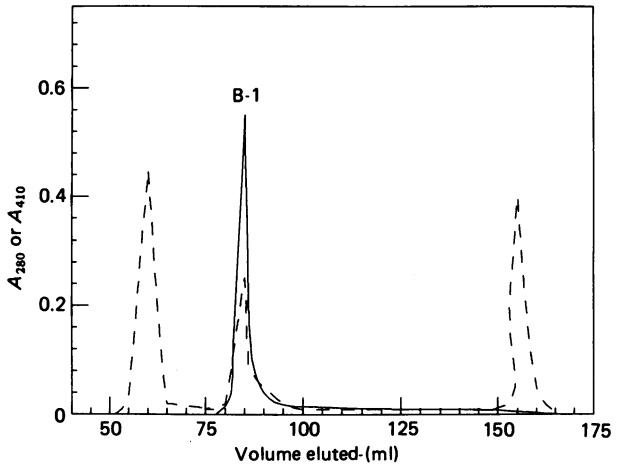

Fig. 5. Gel-filtration pattern showing the tryptic digest of the small CNBr-cleaved fragment of Hnb-modified avidin

Fraction B (see Fig. 3) of the Sephadex-G-50-chromatographed CNBr-cleaved Hnb-modified avidin was digested with trypsin for 20 h as detailed in the text. The digest was subjected to gel filtration on a Sephadex G-25 column, and the eluent was monitored as outlined in the legend to Fig. 4: -----, A_{280}; ——, A_{410}.

residue was modified, the peptide was digested with trypsin under the conditions described above. Gel filtration on Sephadex G-25 (Fig. 5) revealed only one Hnb-containing peak (fraction B-1), the amino acid analysis of which revealed that Trp-110 was modified (Table 1). Quantitative determinations indicated that the latter corresponds to about 50% of the total Hnb modification in avidin.

DISCUSSION

During the course of our studies on the involvement of individual amino acid residues in strong binding ($K_a = 10^{15}$ M^{-1}) between the protein avidin and the vitamin biotin, we were interested in determining the involvement of tryptophan in the binding. Tryptophan has previously been implicated from various spectroscopic studies in connection with chemical modifications of avidin.

In a review on avidin that summarized more than a decade of intensive study, Green (1975) asked three questions with regard to the role of tryptophan in the binding of biotin. These are: (1) how many of the four tryptophan residues interact directly with bound biotin?; (2) is it possible to modify them selectively?; (3) is any of particularly vital importance? In our present study, we have succeeded in answering these questions, and, even more importantly, to determine which tryptophan residues in the protein sequence are involved in the binding.

It is clear from the present study that two tryptophan residues (70 and 110) are modified selectively and are involved in the binding of biotin to avidin. The facts that only one Hnb group binds covalently to the avidin and attaches to two different tryptophan residues, and that the modification of any one of these residues inhibits biotin binding, indicate strongly that these residues must be located in the region of the binding site of avidin that interacts with the biotin moiety.

The only other amino acid residue that has been implicated as part of the binding site is lysine. We have previously shown that, upon reaction of avidin with 1-fluoro-2,4-dinitrobenzene (Gitlin *et al.*, 1987) one dinitrophenyl group is introduced per subunit of avidin, with total inhibition of biotin binding. The dinitrophenyl groups are distributed on three different lysine residues namely Lys-45, Lys-94 and Lys-111. It may be noted that dinitrophenyl-modified avidin is resistant to Hnb-modification conditions (G. Gitlin, E. A. Bayer & M. Wilchek, unpublished work).

The involvement of these two amino acid residues in the binding site can also be deduced from homology found with the now-sequenced streptavidin (Argarana *et al.*, 1986), a bacterial protein that binds biotin with a similar affinity. In this protein, two Trp-Lys sequences (79–80 and 120–121) are conserved, which appear in positions analogous to those (70–71 and 110–111) in egg-white avidin. The present study confirms the involvement of these same tryptophan residues in biotin binding of avidin, since Trp-70 and Trp-110 were modified selectively. Studies with streptavidin with the same reagents are now required.

From the knowledge of the structure of biotin, we would like to speculate that the biotin molecule may be arranged in the binding site in such a way that its carboxy group turns towards the lysine residues, forming an ion-pair interaction with one of the amino groups of the lysine residues, whereas the ureido and thiophan rings of biotin turn towards the indole ring of tryptophan, forming hydrogen bonds or other weak bonds.

The binding of biotin to proteins, particularly the avidin–biotin interaction, is an ideal prototype system in which to study the foundations of binding sites in general, since there are available many biotin-binding proteins with different affinities, e.g. streptavidin, antibodies to biotin, biotin receptor and the enzyme biotinidase, which removes biotin from peptides. Use of chemical and physical methods, such as chemical modification, affinity labelling, X-ray crystallography and site-directed mutagenesis, and the comparison of the homologies in the respective binding sites, will, we hope, teach us about the structure–function relationship of binding sites in general.

This study was supported in part by the Israel Ministry of Science and Development, the National Council for Research and Development, and by the U.S.–Israel Binational Science Foundation (B.S.F.), Jerusalem, Israel.

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Received 14 September 1987; accepted 28 October 1987