Studies on the biotin-binding site of avidin

Lysine residues involved in the active site

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Egg-white avidin was treated with 1-fluoro-2,4-dinitrobenzene. Modification of an average of one lysine residue per avidin subunit caused the complete loss of biotin binding. Tryptic peptides obtained from the 2,4-dinitrophenylated avidin were fractionated by reversed-phase h.p.l.c. Three peptides contained the 2,4-dinitrophenyl group. Amino acid analysis revealed that lysine residues 45, 94 and 111 are modified and probably comprise part of the biotin-binding site.

INTRODUCTION

Avidin is a basic glycoprotein, found in the egg-white and tissues of birds, reptiles and amphibia (Elo & Korpela, 1984). The protein contains four identical subunits having a combined molecular mass of 67000 Da (Green, 1975). The subunit has been sequenced and consists of a single polypeptide chain containing 128 amino acid residues (De Lange & Huang, 1971).

The avidin–biotin interaction ($K_D = 10^{-15}$ M) is the strongest known biochemical (non-covalent) bond. The vitamin biotin is a well-defined molecule of relatively simple structure and of ubiquitous distribution in Nature. For many years, the uniqueness of this strong interaction has puzzled scientists, and, consequently, the avidin–biotin complex has been the subject of intensive study and applications.

Early experiments (Fraenkel-Conrat *et al.*, 1952), in which the effect of chemical modification procedures on the activity of avidin was studied, led to the conclusion that the biotin binding was insensitive to extensive substitution of a variety of functional groups. However, through the years, two types of amino acid have been implicated in the avidin–biotin interaction. Biotin-induced difference spectra (Green, 1963) and chemical modification studies (Green, 1962, 1975) indicated that tryptophan plays an important role in biotin binding. The other functional group that has been directly implicated in the binding site of avidin is an amino group. In this regard, it was observed (Green, 1975) that the initial rate of reaction of 1-fluoro-2,4-dinitrobenzene with avidin was faster than with the avidin–biotin complex. If the reaction was stopped after an average of one 2,4-dinitrophenyl (Dnp) group had been introduced per subunit, almost no biotin-binding activity remained (Green, 1975), suggesting the involvement of a lysine residue in the active site. The precise location of this lysine residue, however, was not determined. In contrast with the interaction of 1-fluoro-2,4-dinitrobenzene with avidin, both 2,4,6-trinitrobenzenesulphonic acid and dansyl chloride reacted with avidin without loss of biotin-binding activity (Green, 1963).

In the general framework of our studies on the understanding and application of the strong interaction

between avidin and biotin (Bayer & Wilchek, 1980; Wilchek & Bayer, 1984), we decided to determine which lysine residue(s) is modified by 1-fluoro-2,4-dinitrobenzene. We were particularly interested in determining which lysine residue(s) is essential to the avidin–biotin interaction. Our results support Green's (1975) initial observation that the modification of only one lysine residue per subunit is sufficient to destroy biotin binding completely. Moreover, this could be accomplished by the modification of any one of three separate lysine residues: Lys-45, Lys-94, Lys-111.

MATERIALS AND METHODS

Materials

Avidin was provided by S. S. Belovo (Bastogne, Belgium). Biotin, bovine serum albumin 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).

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 Dnp-modified avidin were applied to the wells of plates coated with biotinylated bovine serum albumin, and subsequent interaction with biotinyl alkaline phosphatase was measured.

Lysine modification

The reaction was performed by 1-fluoro-2,4-dinitrobenzene at pH 9.2 with ratios of reagent ranging from 0.2:1 to 3.0:1 with respect to the molar concentration of the avidin subunit. The reaction was carried out at 40 °C for 1 h in the dark, after which the solution was dialysed against water. For preparative work, a 50 mg sample of avidin was treated with 1.12 mg of 1-fluoro-2,4-dinitrobenzene. In this case, the extent of Dnp-modified lysine per subunit of avidin was determined (spectrophotometrically, $\epsilon_{360} = 17000 \text{ M}^{-1} \cdot \text{cm}^{-1}$) to be 0.94.

Abbreviation used: Dnp, 2,4-dinitrophenyl.

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Tryptic hydrolysis

The Dnp-modified avidin was boiled for 5 min, after which trypsin (0.5 mg) was added. The hydrolysis was carried out for 20 h at 40 °C in 2 ml of 0.05 M-NaHCO₃, pH 8.0. The reaction mixture was freeze-dried and dissolved in 5% formic acid. Fractionation of the resultant peptides was accomplished by reversed-phase h.p.l.c. under the following conditions. A Lichrosorb RP-18 (E. Merck, Darmstadt, Germany) column (250 mm × 4 mm) was used for separation. Solvents A and B comprised 0.1% (v/v) trifluoroacetic acid in water and 0.1% (v/v) trifluoroacetic acid in 50% (v/v) propan-1-ol respectively. The separation was carried out within 50 min with a linear gradient ranging from 20% (v/v) solvent B (80% solvent A) to 80% (v/v) solvent B. The respective fractions from each Dnp-containing peak were pooled, the extent of Dnp-Lys was determined spectrophotometrically, and the corresponding amino acid content was determined by amino acid analysis as described below.

Amino acid analysis

The amino acid composition 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 Analyzer (Durrum Instrument Corp., Palo Alto, CA, U.S.A.). Tryptophan was determined after hydrolysis in 4 M-methanesulphonic acid under vacuum at 110 °C for 20 h.

RESULTS

Avidin was modified by 1-fluoro-2,4-dinitrobenzene with a slight excess of the reagent. The absorbance spectrum of the Dnp-modified avidin is presented in Fig. 1. The extent of modification did not exceed one group per subunit of protein, and only ϵ -amino groups of lysine were modified, as determined by amino acid analysis and t.l.c. Avidin was treated with various amounts of the reagent, and, correspondingly, different quantities of Dnp were incorporated into the protein, as determined

Fig. 1. Spectrum of Dnp-modified avidin

Solutions (0.17 mg/ml) of Dnp-modified (—) or native (---) avidin were examined on a Cary model 14 spectrophotometer.

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

The percentage of activity remaining after modification (compared with the activity of the native protein) is plotted against the number of modified lysine residues. Modification of one residue per subunit incurred 100% loss in the activity.

Fig. 3. H.p.l.c. pattern of the tryptic digest of Dnp-modified avidin

Dnp-modified avidin was subjected to 24 h interaction with trypsin as detailed in the text. A portion of the digest was chromatographed on a Lichrosorb RP-18 column with a linear gradient containing 50% (v/v) propan-1-ol (solvent B) in 0.1% trifluoroacetic acid (·····). Peptide elution was monitored by absorbance at 280 nm (----) and 360 nm (—).

spectrophotometrically. The biotin-binding activity of avidin decreased upon increasing the extent of modification of lysine residues. By graphing the results according to Tsou (1962), the contribution of the modification to inactivation of avidin could be determined (Fig. 2). The results show that modification of one lysine residue per subunit is sufficient to cause complete loss of biotin binding.

Table 1. Amino acid composition of avidin peptides containing ϵ -Dnp-lysine

The values were normalized to alanine and are represented on the basis of five alanine residues/avidin subunit. The values in parentheses represent the actual number of amino acid residues in the ϵ -Dnp-Lys-containing peptides of avidin. The extent of ϵ -Dnp-Lys was determined spectroscopically.

Amino acid	Amino acid composition (residues/molecule of peptide)		
	Peak T1 (residues 101–114)	Peak T2 (residues 27–58)	Peak T3 (residues 91–100)
Asx	3.6 (4)	2.5 (3)	
Thr	0.8 (1)	6.4 (7–8)*	0.8 (1)
Ser	1.6 (2)	1.5 (2)	
Glx		3.2 (4)	1.2 (1)
Pro		0.9 (1)	
Gly	1.1 (1)	2.6 (3)	
Ala	1.0 (1)	2.0 (2)	
Met			1.0 (1)
Val	0.9 (1)	1.3 (1)	1.0 (1)
Ile	0.8 (1)	1.7 (2–3)*	
Leu		1.0 (1)	1.7 (3)†
Trp	1.0 (1)		1.0 (1)
Tyr		0.7 (1)	
Phe		1.1 (1)	
His		0.9 (1)	
Lys		1.0 (1)‡	
Arg	0.8 (1)		0.8 (1)
ϵ -Dnp-Lys	1.0	1.0	1.0

* There is some heterogeneity in position 33 of the native avidin molecule, where isoleucine is replaced by threonine.

† Incomplete hydrolysis of the Leu–Leu bond (position 98–99) causes lower leucine values.

‡ The lysine indicated is C-terminal.

A sample of Dnp-modified avidin was subjected to tryptic cleavage, and the resultant peptides were separated by reversed-phase h.p.l.c. The elution profile is illustrated in Fig. 3. The chromatogram shows four Dnp-containing peaks. The corresponding primary structure of each peak was determined by amino acid analysis, and the extent of Dnp-modified lysine was determined spectroscopically. As shown in Table 1, peak T1 (which contains about 38% of the Dnp incorporated into avidin) corresponded to the peptide that included Lys-111. Peak T2 contained Lys-45 (about 18% of the total label). Peak T3 was the major Dnp-containing fraction (about 44%) and represents the tryptic peptide that contains Lys-94. The fourth peak does not contain detectable quantities of amino acid and probably represents some adsorbed Dnp.

DISCUSSION

The binding of biotin to avidin is the strongest interaction between a ligand and a protein known in Nature. By understanding the nature of this interaction and by elucidating the structure of the biotin-binding site in avidin, we may be able to explain better how Nature 'plans' and produces binding sites in general.

The identification of the lysine residues, the modification of which abolishes the strong binding between biotin and avidin, is a step towards the elucidation of the stereochemistry and functional organization of residues at or near the binding region of avidin. The fact that 1-fluoro-2,4-dinitrobenzene attaches to three different lysine residues (Lys-45, Lys-94 and Lys-111), the modification of any of which inhibits biotin binding, indicates that all three of these residues must be located in the region of the biotin-binding site of avidin.

Reaction at one position in the active site excludes reaction at other lysine residues, a phenomenon that confirms the specificity of the reaction. Dnp, once irreversibly bound to one of the lysine residues, may block access to this region for biotin or other Dnp molecules. For this reason it cannot be said unequivocally that the lysine residues that react with 1-fluoro-2,4-dinitrobenzene are, in themselves, essential for binding. These may serve simply as attachment sites that fasten the Dnp reagent firmly at the binding site. The only other amino acid residue that has been implicated as part of the binding site is tryptophan (Green, 1975). The involvement of these two amino acid residues in the binding site can also be deduced from the homology found with the recently sequenced streptavidin (Argarana *et al.*, 1966), 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.

Therefore, one of the most surprising findings of the present study is that most of the labelling occurs on Lys-94 (44%), which is not positioned next to a tryptophan residue in the sequence. This indicates that, in the tertiary structure of avidin, this particular lysine may be near the tryptophan residue(s) (70 and/or 110), similar to the adjacent lysine(s) in the sequence (residues 71 and 111). Alternatively, Lys-94 may occupy a more exposed position near the binding site. The other surprising result is that Lys-71 was not labelled, despite the speculation that it forms part of the binding site (Argarana *et al.*, 1986).

More information concerning the binding site needs to be obtained by using cross-linking reagents (e.g. 1,5-difluoro-2,4-dinitrobenzene), affinity labels containing biotin and X-ray crystallography of avidin both with and without biotin. The comparison of these results with similar studies on streptavidin should confirm their general relevance towards biotin binding.

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