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# Monoclonal anti-biotin antibodies simulate avidin in the recognition of biotin

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The sequence of the $V_H$ gene of a monoclonal anti-biotin antibody was determined. Biotin-binding motifs, similar to those in avidin and streptavidin, were identified in complementarity determining regions 2 and 3, suggesting that natural selection of functional motifs may occur in unrelated protein types.

Anti-biotin antibody; Antibody gene; Biotin binding site; Antibody combining site; Binding motif

## 1. INTRODUCTION

The avidin–biotin complex is the epitome of biological recognition processes [1,2]. Knowledge of the interactions which participate in the formation of this complex may eventually lead to a better understanding of the interaction between proteins and ligands in general.

Recently, we have succeeded in elucidating the three-dimensional structure of egg white avidin and its complex with biotin [3]. Comparison with the known structure of its bacterial relative, streptavidin [4,5], has revealed that the overall fold of the two proteins is very similar. The close similarity also applies to most of the residues in the respective binding sites which are crucial for interaction with the biotin molecule. Interestingly, the similarity in binding can be traced to individual amino acid residues which occupy defined positions in six homologous stretches of the two proteins [6,7]. Thus, although the overall homology in the primary structures of avidin and streptavidin is only 30% (43% similarity), the extent of homology within these homologous stretches is 65% (80% similarity).

These findings have prompted us to consider whether such a high-affinity interaction is simply an accident of nature or whether it reflects the product of evolutionary design. In this context, the biotin binding sites of avidin and streptavidin were contrived throughout an evolutionary time-frame of millions of years. It was of interest to us to consider a contrasting situation, where a biotin binding site exists in a different type of molecule, e.g. an antibody. When the biotin moiety is covalently attached to a foreign carrier protein, anti-biotin antibodies are elicited. We thus questioned whether the immune system would select the same type of elements (i.e. the same amino acids) to produce a biotin binding site in an antibody as those which are known to recognize biotin in the binding site of avidin.

In order to address this question, we produced monoclonal anti-biotin antibodies, which bind the vitamin with a relatively high affinity ($K_A \sim 10^9\ M^{-1}$). We report here the cloning and sequencing of the $V_H$ cDNA of this antibody. Comparison of the sequence of $V_H$ with those of avidin and streptavidin revealed an astonishing similarity in the CDR2 and CDR3 regions of the antibody with known biotin binding motifs in two of the homologous stretches of avidin and streptavidin.

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## 2. MATERIALS AND METHODS

### 2.1. *Reagents and solutions*

Tris-(hydroxymethyl)-aminomethane, bovine serum albumin (BSA, RIA grade), Tween 20, diethylene triamine pentaacetic acid (DTPA), bovine γ-globulin, incomplete and complete Freund's adjuvant, 1-ethyl-3(3-dimethyl aminopropyl) carbodiimide (WSC, water-soluble carbodiimide), poly-L-lysine ($M_r$ 15,000–30,000) and ovalbumin were purchased from Sigma Chemical Co. (St. Louis, MO). Affinity-purified rabbit anti-mouse immunoglobulin was obtained from Dakopatts, Glostrup, Denmark. Sepharose-protein A and Sephadex G-25 were products of Pharmacia, Uppsala, Sweden. Anti-mouse immunoglobins (used for Ouchterlony immunodiffusion) were from Serotec (Oxford, UK). The labeling reagent [*N*-1-(*p*-isothiocyanatophenyl) diethylene triamine-$N^1$,$N^2$,$N^3$-tetraacetate (DTTA)], chelated with $Eu^{3+}$, was kindly provided by Dr. Iikka Hemmila, Wallac Oy, Turku, Finland.

Coating buffer for ELISA plates consisted of 50 mM sodium carbonate, pH 9.6, containing 0.02% sodium azide. Time-resolved fluo-

rescence was measured using the enhancement solution obtained from Wallac, Oy. The assay buffer was 50 M Tris-HCl buffer, pH 7.75, containing BSA (5 g/l), bovine $\gamma$-globulin (0.5 g/l), 20 $\mu$mol of DTPA, sodium chloride (9 g/l), Tween 20 (0.5 ml/l) and sodium azide (0.5 g/l).

Biotinylated BSA was prepared using biotin *N*-hydroxysuccinimide ester [8].

2.2. *Preparation of europium-labeled reagents*

Biotin was conjugated to polylysine using the WSC coupling procedure [8]. The resultant derivative (1 mg/ml) was dialyzed for 2 h against 50 mM carbonate buffer, pH 9.8, and derivatized further with europium-chelated DTTA, as described previously [9].

2.3. *Preparation of monoclonal anti-biotin antibodies*

Female, 2-month-old CB6/black mice were immunized with biotinylated BSA (50 $\mu$g/mouse) in complete Freund's adjuvant. Subsequently, two booster injections were given, using the biotinylated BSA preparation in incomplete Freund's adjuvant. After a 2-month immunization period, the antibody titer was checked using rabbit anti-mouse IgG-coated plates (prepared as described previously [10], europium-chelated biotin–polylysine as a label, and time-resolved fluorescence as an end point [9,10]). Three months after the initial immunization, spleen cells of the mouse were selected on the basis of their interaction with the europium-chelated biotin–polylysine preparation. Those which exhibited the highest serum titer of antibody which recognized the biotin-containing label were fused with a mouse myeloma cell line (NSO, kindly donated by Dr. C. Milstein, Cambridge), using the hybridoma technique of Köhler and Milstein [11]. The culture supernatants of growing hybridomas were screened for antibody activity using rabbit anti-mouse IgG-coated plates, and the europium-chelated biotin–polylysine. Four hybridomas were selected; the clone (F1) which demonstrated the highest affinity for biotin was propagated in vitro and used to prepare RNA.

2.4. *Cloning of the gene for the $V_H$ region of monoclonal anti-biotin*

The $F_1$ hybridoma cell line which secreted anti-biotin antibodies ($IgG_1$) was grown in Dulbecco's modified Eagle's medium, supplemented with 10% (v/v) horse serum. Total cellular RNA was prepared from $F_1$ cells ($5 \times 10^6$) using the Nonidet P-40/SDS technique as described by Gough [12]. Poly(A)-containing RNA was purified by affinity chromatography over magnetic oligo(dT) [13]. First-strand DNA synthesis was performed by incubating poly(A) mRNA (~ 0.1 $\mu$g) at 37°C for 90 min with a mixture of 50 mM of Tris-HCl, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, RNase inhibitor, 1 mM deoxynucleotides, 2.5 ng of oligo$(dT)_{15}$ as primer and AMV reverse transcriptase in a reaction volume of 60 $\mu$l. 10 $\mu$l of the cDNA reaction mixture were used directly in a PCR, using 2.5 U *Taq* DNA polymerase with 25 pmol of the mouse $V_H$ forward primer (5′-TGAGGAGACGGTGACCGTGGTCCCTTGGCCCC) and 25 pmol of the mouse $V_H$ backward primer (5′-AGGT(C/G)(A/C)A(A/G)CTGCAG(C/G)AGTC(A/T)GG), and deoxynucleotides in *Taq* buffer [14]. The mixture was amplified for 30 cycles and an ethidium bromide-stained 2% agarose gel was used to visualize the PCR fragments. The amplified product was purified by electroelution, and blunt ended with Klenow enzyme and $T_4$ polynucleotide kinase. The DNA product was inserted into pBluescriptKS$^-$ vector which was digested with *Sma*I and dephosphorylated. The ligation mixture was used to transform *E. coli* TG1 competent cells, and transformants were selected on LB plates, containing ampicillin, IPTG and X-Gal. From the resulting colonies, DNA minipreparations were performed; the size of the inserts were analyzed by an *Xba*I/*Sal*I digestion. Clones containing inserts of the correct size (approximately 350 bp) were grown in 5 ml TB medium. Following growth, cells were collected by centrifugation and lysed. The supernatant was then decanted into a Magic minicolumn (Promega), and plasmid DNA was eluted according to the procedure recommended by the manufacturer. The purified DNA (approximately 0.5 $\mu$g) was sequenced on an ABI 373 DNA sequencer (Applied Biosystems Inc.), using SK or KS primer and cycle-sequencing reactions [15].

## 3. RESULTS AND DISCUSSION

In this study, a biotin-specific monoclonal antibody was elicited, its $V_H$ gene was cloned and sequenced. The sequence is presented in Fig. 1. Upon comparing this sequence with those of avidin and streptavidin, we noticed that the CDR2 and CDR3 regions of the antibody show a startling resemblance to two homologous stretches in avidin and streptavidin which are known to interact with the bicyclic ring system of biotin (see Fig.

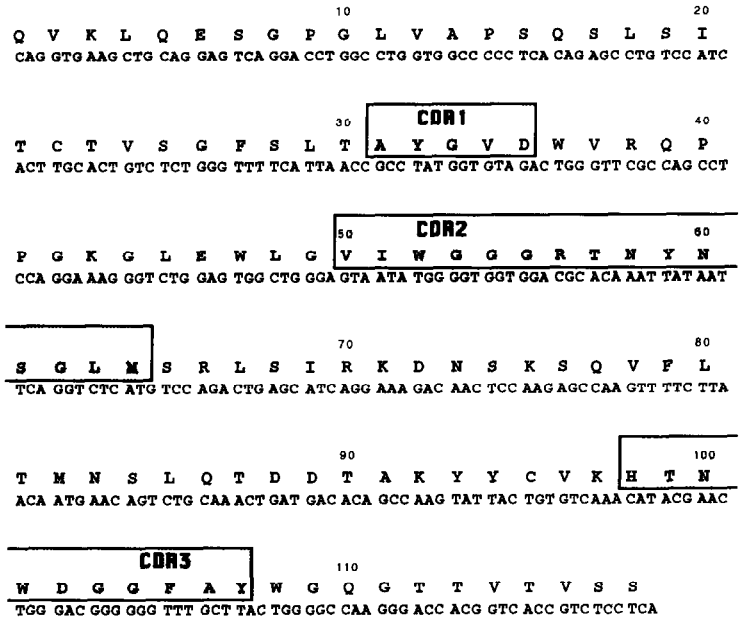


Fig. 1. Nucleotide and deduced amino acid sequence of the $V_H$ gene of the anti-biotin antibody. Single-letter amino acid codes are used. The complementarity-determining regions are indicated by shaded areas. The numbering is according to Kabat et al. [16].

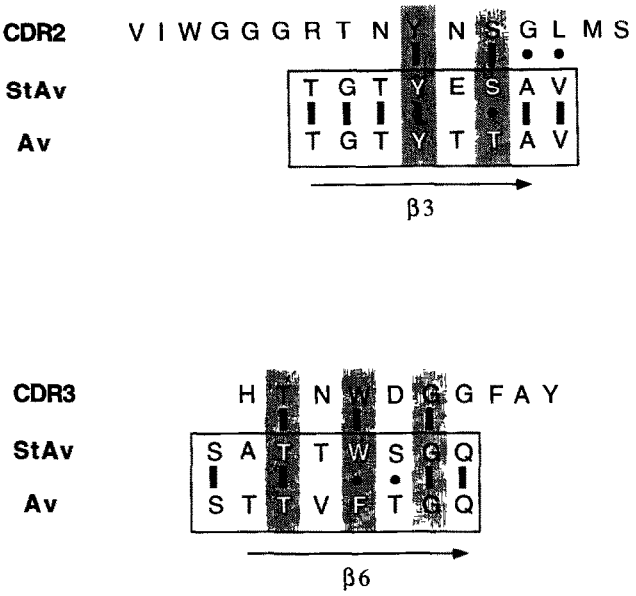


Fig. 2. Alignment of the amino acid sequences of complementarity-determining regions 2 and 3 of the anti-biotin antibody with relevant homologous stretches of avidin (Av) and streptavidin (StAv). Identical residues are denoted by vertical bars and similar conserved residues are indicated by dots. Identity in the antibody is emphasized by shading. Amino acid residues in the binding sites of avidin and streptavidin, which are known to interact with the bicyclic ring of the biotin molecule, are shown in white lettering. The second and fourth homologous stretches of avidin and streptavidin encompass the $\beta$3 and $\beta$6 strands of the two proteins.

2). Specifically, the second homologous stretch of avidin and streptavidin (which encompasses the $\beta$3 strand of both proteins) contains the sequence xxxYxT(S)xx, and the CDR2 of the heavy chain of the antibody contains RTNYNSGL. Similarly, the fourth homologous stretch of avidin and streptavidin (which encompasses the $\beta$6 strand) contains the sequence xxTxF(W)xG, and the CDR3 of the antibody heavy chain contains HTNWDG. These results seem to indicate a similar pattern in the sequences which may dictate the quality of binding biotin.

A comparison of the sequences of the complementarity-determining regions of different antibodies, deposited in the SWISS-PROT protein data bank (Release 23), revealed that the sequence, YNS, is very common in the CDR2 of the heavy chain. On the other hand, we could not find the sequence TxWxG in any other CDR3 of known antibody sequence.

These findings may indicate two separate principles. On the one hand, nature does not always have to produce new functional motifs in order to produce a binding site, since the YxS theme of the CDR2 is adequate for binding the ureido ring of the biotin moiety. Indeed, in both avidin and streptavidin, this particular conserved tyrosine-containing stretch has been shown to be decisive for the binding of biotin [17,18]. On the other hand, a new motif was introduced into the CDR3 of the antibody, presumably to reinforce the binding. Interestingly, in both avidin and streptavidin, this conserved motif is located near the thiophene ring of biotin; if future X-ray analysis of the antibody confirms the presence of such a set of interactions, we may conclude that in nature such functional elements can be used for a given purpose irrespective of the protein type (i.e. avidin or antibody).

This phenomenon may thus be an example of convergent evolution at the molecular level. The veracity of this notion is supported by the production of monoclonal antibodies against the fibrinogen binding receptor on platelets, in which the CDR3 of the heavy chain contains an RYD sequence which mimics the RGD motif of various adhesion proteins of the extracellular matrix [19]. These results also underscore the salience of the CDR3 in antigen recognition.

Finally, our findings may pave the way for a new approach to engineering of specific combining sites on antibodies. Known binding sites from other proteins can thus be transferred to an appropriate CDR region of an appropriate antibody. For example, the 10–12 residue bungarotoxin binding site of the acetylcholine receptor has been incorporated into a chimeric protein from *E. coli*, and the resultant protein exhibits bungarotoxin binding activity [20]. Insertion of this peptide into the CDR3 of the $V_H$ of a carrier immunoglobulin may thus impart the quality of bungarotoxin binding in an antibody. Likewise, catalytic antibodies may be produced by transferring known binding and catalytic sites from enzymes to the CDRs of an antibody molecule.

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