Mutation of the important Tyr-33 residue of chicken avidin: functional and structural consequences

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The strong interaction between avidin and biotin is so tight (dissociation constant 10^{-15} M) that conditions usually sufficient for protein denaturing fail to dislodge biotin from the avidin–biotin complex. This kind of irreversible binding hinders the use of avidin in applications such as affinity purification or protein immobilization. To address this concern, we have constructed a series of mutants of the strategically positioned Tyr-33 in order to study the role of this residue in biotin binding, and to create avidin variants with more reversible ligand-binding properties. Unexpectedly, an avidin mutant in which Tyr-33 was replaced with phenylalanine (Avm-Y33F) displayed similar biotin-binding characteristics to the native avidin, indicating that the hydrogen bond formed between the hydroxy group of Tyr-33 and the carbonyl oxygen of biotin is not as important for the tight binding of biotin as previously suggested. In terms of the

reversibility of biotin binding, Avm-Y33H was the most successful substitution constructed in this study. Interestingly, the binding of this mutant exhibited clear pH-dependence, since at neutral pH it bound to the biotin surface in an irreversible fashion, whereas, at pH 9, 50% of the bound protein could be released with free biotin. Furthermore, although Tyr-33 is located relatively distant from the monomer–monomer interfaces, the mutagenesis of this residue also weakened the quaternary structure of avidin, indicating that the high ligand binding and the high stability of avidin have evolved together and it is difficult to modify one without affecting the other.

Key words: Avidin–biotin technology, biotin-binding protein, reversibility of binding, shape complementarity, structure–function relationship.

INTRODUCTION

The biotin-binding proteins, chicken avidin and bacterial streptavidin, are interesting proteins at least in two respects. First, the affinity that these two proteins display when binding biotin [K_d (dissociation constant) $\sim 10^{-15}$ M] is among the highest known in nature between a protein and a ligand [1]. The (strept)avidin–biotin complex can thus serve as a model for protein–ligand interactions and for the study of the involvement of individual amino acid residues in the binding site. Secondly, this strong interaction between avidin (and streptavidin) and biotin has been utilized in a plethora of different applications in the life sciences [2,3]. However, in some applications, like affinity chromatography or protein immobilization, the irreversible nature of the (strept)avidin–biotin complex can be a severe hindrance, since extreme denaturing conditions are required for disrupting this complex, usually leading to inactivation of the biotinylated molecule. Therefore, it would be useful to have avidin (or streptavidin) variants that would still bind biotin with high affinity but wherein the binding could be reversed by either adding free biotin or by changing the binding conditions.

Based on the X-ray crystal structures of avidin [4–6] and streptavidin [7,8], three major interactions contribute to their extremely tight biotin binding: (i) van der Waals interactions between biotin and the aromatic side chains of the binding-site residues, (ii) an extensive H-bonding network between the protein and the ligand, and (iii) the re-organization of a flexible L3,4 loop upon biotin binding. Previous studies both with avidin [9] and streptavidin [10,11] have shown that changing the hydrophobic amino acids in the binding site often leads to drastically lowered affinity and to distortion of the quaternary structure.

However, since the effects of individual H-bonds are usually relatively small compared with the overall binding energy, site-directed mutagenesis of avidin residues forming H-bonds with biotin might enable fine-tuning of the strength of the avidin–biotin complex.

Chicken avidin has only one tyrosine (Tyr-33) residue, whereas streptavidin has six tyrosines, one of which (Tyr-43) is equivalent to the single tyrosine residue of avidin. This tyrosine in both avidin and streptavidin is located in an 8-residue conserved stretch, and a synthetic peptide based on this stretch has been shown to bind biotin, albeit with low affinity [12]. According to the three-dimensional structures [4–8], this conserved tyrosine forms one of the H-bonds in the hydrogen bond network (Figure 1), which has been proposed to focus complementary electrostatic interactions with the polarized ureido oxyanion of biotin and could thus make a relatively significant energetic contribution to the (strept)avidin–biotin complex [4,13]. Consequently, Morag et al. [14] have shown that reversible biotin binding can be achieved by chemical modification of this conserved tyrosine residue in both avidin and streptavidin.

In the present study, we examined whether a single H-bond can have such a profound effect on the binding of a ligand to a protein or whether other factors, e.g. structural, should also be considered. In this context, we report here the construction of four different avidin mutants, which were designed to address the involvement of the critical tyrosine residue. In the avidin mutant in which Tyr-33 was replaced with phenylalanine (Avm-Y33F), the hydroxy group was removed, but the aromatic (hydrophobic) character of the residue was retained. In the avidin mutant in which Tyr-33 was replaced with alanine (Avm-Y33A), the entire aromatic group, together with its hydroxyl substituent, was

Abbreviations used: Avd, native avidin; Avm-Y33F/Q/A or H, avidin mutant in which Tyr-33 is replaced with phenylalanine/glutamine/alanine or histidine; H-bond, hydrogen bond; K_d , dissociation constant; k_{ass} , association rate constant; k_{diss} , dissociation rate constant.

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Figure 1 H-bonding network between the binding-site residues of avidin and the ureido oxygen of the biotin

Asn-12, Ser-16 and Tyr-33 of avidin participate in H-bonding interactions with the polarized carbonyl oxygen of biotin. Streptavidin forms an essentially identical network involving Asn-23, Ser-27 and Tyr-43.

removed. The avidin mutant in which Tyr-33 was replaced with glutamine (Avm-Y33Q) lacked the hydrophobic property of the aromatic group but enabled the formation of an alternative H-bond with the ligand. Finally, the avidin mutant in which Tyr-33 was replaced with histidine (Avm-Y33H) retained partial aromatic properties with the potential for pH-dependent H-bond formation. The effects of these mutations on biotin binding, stability characteristics and subunit interactions in avidin are discussed.

EXPERIMENTAL

Construction of avidin mutants and preparation of recombinant baculoviruses

Site-directed mutagenesis of avidin cDNA [15] was carried out with PCR using the Megaprimer method, as described previously [16]. The mutations were confirmed by double-stranded sequencing using Sanger's dideoxynucleotide chain termination procedure with an automated DNA sequencer [ALF (Pharmacia Biotech) or DNA Analyzer Gene Reader 4200 (Li-Cor, Lincoln, NE, U.S.A.)]. The recombinant baculoviruses were generated using the Bac-To-Bac baculovirus expression system according to the manufacturer's instructions (Gibco BRL). The primary virus stocks were amplified for large-scale production of avidin mutants and the titres of the stocks were determined by a plaque assay procedure [17].

Production and purification of the avidin mutants

The mutant proteins were produced in baculovirus-infected Sf9 insect cells as reported previously [16]. Biotin-free medium was used for expression, since, if biotin is present, it will block the binding sites of avidin variants, hampering subsequent affinity purification. After production, the proteins were purified with affinity chromatography using 2-iminobiotin agarose, as described previously [18].

Protein analysis

Many of the comparative assays used in this work have been described in previous publications [9,16,19,21]. The quaternary

status of avidin and mutant proteins was analysed by FPLC either on a Superose 12 column (10 cm × 30 cm; Pharmacia) using an LKB HPLC system or by using a Shimadzu HPLC system with a Superdex TM 200 HR (10 cm × 30 cm) column. A sample [5–40 µg in 20–100 µl of phosphate buffer with 0.65 M NaCl (pH 7.2), or 50 mM sodium acetate with 0.65 M NaCl (pH 4) or 50 mM Na-carbonate with 0.65 M NaCl (pH 11)] was applied, and chromatography was carried out at a flow rate of 0.5 ml/min [16]. The thermal stability characteristics of the avidin mutants were assessed electrophoretically, as described previously [19]. Protease sensitivity was determined according to Laitinen et al. [9] and electrophoretic analysis was carried out using SDS/15% (w/v) PAGE in a discontinuous buffer system [20].

Biotin-binding assays

Binding kinetics were measured with optical biosensor technology (IASys Manual+; Affinity Sensors, Cambridge, U.K.) using either biotin or 2-iminobiotin cuvettes, as reported previously [16]. Reversibility of the biotin binding was determined by competitive binding to a biotinylated biosensor surface according to the methods of Laitinen et al. [9].

RESULTS

Production and purification of mutated avidins

The avidin mutants were produced in baculovirus-infected Sf9 insect cells, and the proteins were purified from cell lysates. In the case of Avm-Y33F, Avm-Y33A and Avm-Y33H, the purification with affinity chromatography using 2-iminobiotin as a capturing ligand resulted in pure products of high yields (results not shown), comparable with those reported previously both for wild-type avidin and several avidin mutants [16,18,21]. With Avm-Y33Q, the binding to 2-iminobiotin was considerably impaired, so that less than 50% of the protein was captured during the chromatographic step.

Figure 2 Reversibility of the biotin-binding activity of wild-type avidin and avidin Tyr-mutants

Reversibility of binding was determined with an IASys optical biosensor by determining the amount of bound protein released from the biotin surface by adding free biotin. Chemically modified nitro-avidin [14] was used as a positive control. The assay was performed in 50 mM borate buffer (pH 9) containing 1 M NaCl. The results are expressed as percentage reversibility (mean ± S.D.).

Table 1 Kinetic parameters for binding of native avidin and different mutants to 2-iminobiotin at pH 9.5

Shown below are the binding properties of avidin and different variants to 2-iminobiotin surface determined using the IAsys optical biosensor. Interaction was measured in 50 mM borate buffer (pH 9.5) containing 1 M NaCl at room temperature. The values for avidin are from Marttila et al. [21].

Protein	$k_{\text{ass}} (\text{M}^{-1} \text{s}^{-1})$	$k_{\text{diss}} (\text{s}^{-1})$	$K_{\text{d}} (\text{M})$
Avd	1.6×10^4	3.1×10^{-4}	2.0×10^{-8}
Avm-Y33F	4.9×10^4	4.0×10^{-3}	8.8×10^{-8}
Avm-Y33A	1.7×10^4	1.8×10^{-2}	1.0×10^{-6}
Avm-Y33H	5.0×10^4	6.8×10^{-3}	1.2×10^{-7}

Biotin-binding properties

Reversibility of the biotin binding of avidin and different mutants was measured using competitive binding to the biotinylated biosensor surface, as described previously [9]. At pH 7, all the avidin mutants constructed in this work behaved similarly to that of the wild-type avidin, displaying virtually irreversible binding to the biotinylated surface (results not shown). However, at elevated pH (pH 9 or higher) Avm-Y33H exhibited substantial reversibility, i.e. approx. 50 % of the bound protein could be released with free biotin (Figure 2). Also Avm-Y33Q displayed some reversibility (29 %) at pH 9, whereas with the two other mutants, Avm-Y33F and Avm-Y33A, the change in pH did not have significant effect on the reversibility of the biotin binding.

To obtain additional information about the effects of these mutations on the binding characteristics of different avidin variants, a 2-iminobiotin surface was used in the optical biosensor measurements. 2-Iminobiotin is a biotin analogue, which binds to avidin with lower, readily measurable affinity and has been shown to be a good reporter for intrinsic (strept)avidin–biotin interactions [10,16,21]. Interestingly, Avm-Y33F and Avm-Y33H exhibited only small reductions in the binding affinity for 2-iminobiotin (4-fold and 6-fold respectively), whereas Avm-Y33A displayed a 50-fold decrease (Table 1). In all cases, the decrease in the affinity as compared with that of the wild-type avidin was mostly due to the faster dissociation rate. With Avm-Y33Q, some specific binding to 2-iminobiotin was also detected but the binding was so weak that we were unable to measure the exact kinetic values. When binding to the biotin surface was measured, the results showed that Avm-Y33Q displayed an over 400 times slower association [k_{ass} (association rate constant) = $1.6 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$] than has been reported for avidin ($k_{\text{ass}} = 7 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$) [22]. However, because of the slow dissociation process we were unable to determine the off-rate from the optical biosensor measurements.

Stability and structural properties of avidin mutants

The thermal stability of the different avidin mutants was tested using an SDS/PAGE assay (Figure 3), as described in the Experimental section. In the absence of biotin, the tetramer–monomer transition of the native avidin occurred at about 50 °C. Of the four mutants, Avm-Y33F behaved similarly to the native avidin whereas the other Avm-Y33 mutants were clearly less stable. Already at room temperature (in the presence of SDS), about 50 % of the Avm-Y33A was found to be monomeric and Avm-Y33H and Avm-Y33Q were almost completely dissociated into monomers. Addition of biotin stabilized the tetrameric structure of both the native avidin and all the variants. However, the biotin-bound forms of Avm-Y33A, Avm-Y33H and Avm-

Figure 3 Comparative thermostability of avidin and avidin Tyr-mutants

Samples of free biotin and biotin-saturated with wild-type avidin or one of the avidin Tyr-mutants were combined with sample buffer containing SDS, incubated at the indicated temperatures for 20 min, and then subjected to SDS/PAGE in 15% acrylamide separating gels. Densitometry tracings of the amount of the bands representing the tetramer versus monomer from each of the Coomassie Blue-stained gels were determined, and the relative amount of tetramer was plotted as a function of temperature.

Y33Q, also exhibited diminished thermal stability when compared with those of the native avidin or Avm-Y33F.

The stability properties of the different mutants were also studied by their susceptibility to proteinase K digestion (Figure 4). Without the bound ligand, avidin was degraded slowly, but the addition of biotin rendered avidin immune to any digestion by proteinase K. Avm-Y33F, Avm-Y33H and Avm-Y33Q exhibited stability characteristics similar to those of the native avidin, whereas Avm-Y33A was clearly less stable in the absence of biotin. However, the introduction of biotin also served to

Figure 4 Sensitivity of wild-type avidin and avidin Tyr-mutants to proteinase K treatment

Protein samples (in 100 mM Tris/HCl buffer, pH 7.8), were mixed with proteinase K at a ratio of 1:50 (w/w), both in the presence and the absence of biotin (BTN), and removed at various time points. The samples were dissolved in sample buffer, boiled for 10 min, and then subjected to SDS/PAGE. The values represent the relative percentage of intact monomer observed in the indicated sample plotted as a function of time.

stabilize this mutant so that 80% of the protein was still intact after a 16-h incubation in the presence of proteinase K.

To examine the status of the quaternary structure of Avm-Y33A, Avm-Y33H and Avm-Y33Q under non-denaturing conditions (i.e. in the absence of SDS), a gel filtration FPLC was performed in three different conditions of pH (pH 4, pH 7.2 and pH 11). The results demonstrated that under these conditions, these proteins behaved similarly to the native avidin, forming stable tetramers both in the absence and in the presence of biotin (results not shown).

DISCUSSION

The high affinity of the avidin-biotin interaction has been exploited to develop a tremendous variety of applications in molecular biology and is commonly known as avidin-biotin technology [2]. For both technical and theoretical considerations, it is interesting to modify avidin and streptavidin, both chemically and biologically (via site-directed mutagenesis) to reduce the affinity of this interaction and to study the effects of the modification on the biotin-binding properties, as well as the physical parameters of the protein.

Recently Morag and co-workers [14] have published results on a chemically modified derivative of avidin, nitro-avidin, which displayed reversible biotin-binding characteristics. However, the nitration reaction of Tyr-33 was not complete, leading to a mixture of different end products. In addition, genetically modified versions of avidin and streptavidin with varying levels of reversible biotin binding have also been described [9,23]. The affinity of these proteins was, however, only in the range of $K_d \approx 10^{-6}$ – 10^{-8} M, and they exhibited either dimeric or monomeric quaternary structures, instead of the preferred tetrameric one. For these reasons, the availability of recombinant avidin or streptavidin variants, that would still possess four high-affinity biotin-binding sites but would be reversible under mild conditions, would be desirable and extend further the usefulness of (strept)avidin-biotin technology.

According to the X-ray crystal structure of avidin [4–6], Tyr-33 donates an important H-bond in the interaction with biotin and forms, together with two other binding site residues (Asn-12 and Ser-16), an oxyanion with the carbonyl oxygen of biotin [12,13]. The corresponding bond in streptavidin is formed by analogous Tyr-43, which interestingly is located in the longest conserved stretch found in these two proteins [24]. Furthermore, as previously stated, it has been shown that nitration of this tyrosine residue in the *ortho* position in both avidin and streptavidin leads to reversible interaction with biotin [14]. In addition, if this residue is chemically modified with *p*-nitrobenzenesulphonyl fluoride, which modifies the hydroxy group of tyrosine, the biotin-binding ability is completely abolished [24]. Based on these observations, we chose Tyr-33 of avidin as our target for mutagenesis in order to create avidin mutants with reduced biotin-binding affinity.

Somewhat surprisingly, the deletion of this H-bond by replacement of Tyr-33 with phenylalanine had minimal effect on the biotin-binding ability, since Avm-Y33F displayed binding characteristics similar to the wild-type avidin (Figure 2 and Table 1). These results strongly suggest that this H-bond is not as essential for the tight binding of biotin, as previously thought. Indeed, in a previous publication, Klump et al. [25] studied the effect of deletion of the analogous H-bond in streptavidin and obtained similar results. Furthermore, it has been previously suggested that the pH-dependent binding of 2-iminobiotin to avidin is due to the fact that, at higher pH, its guanido group can form the H-bond with the hydroxy group of Tyr-33, whereas at lower pH, this group is ionized and is unable to form this bond [26,27]. However, in the light of these results, it seems that additional factors must also exist that would account for the observed pH-dependent binding, since simple elimination of this one H-bond would not seem to be enough to dislodge biotin from the binding site of avidin. In the case of streptavidin, it has been suggested [28] that at low pH the loss of the H-bond between the 2-iminobiotin nitrogen atom and Asn-23, as well as the fact that Ser-27 is forced to form more unfavourable H-bonding, may also contribute to the decreased affinity, and similar changes are likely to occur in avidin also.

Figure 5 The pH-dependent interaction between His-33 of Avm-Y33H and the ureido oxygen of biotin

At acid pH, an H-bond is formed between the histidine residue and the polarized carbonyl oxygen of biotin, whereas at higher pH, the bond is lost due to the deprotonation of the imidazole ring, thus promoting reversible binding.

In order to study further the importance of Tyr-33 in the binding of biotin, three other mutants were constructed: Avm-Y33A, Avm-Y33Q and Avm-Y33H. The idea behind Avm-Y33A was to abolish the contribution of the aromatic ring structure of Tyr/Phe to the hydrophobic cage surrounding biotin in the binding pocket of avidin. Indeed, this substitution resulted in clearly measurable changes in the binding characteristics when compared with those of the Avm-Y33F (Table 1) but the binding to biotin was still practically irreversible (Figure 2). In the case of Avm-Y33Q, the affinity towards 2-iminobiotin was severely weakened, as suggested by our unsuccessful attempts to measure kinetic parameters for its binding to this ligand using the IAsys biosensor. The reduced affinity of Avm-Y33Q is further supported by its poor recovery after the affinity purification using the same analogue as a capturing ligand. When binding of this mutant to the biotin surface was measured, a significantly reduced association rate was observed, when compared with that reported for native avidin. Unfortunately, due to the slow dissociation, we were unable to determine the off-rate for binding of Avm-Y33Q to the biotin surface. However, since current biosensor technology allows measurements of off-rates as low as $1 \times 10^{-5} \text{ s}^{-1}$, a maximum K_d for this binding can be estimated to be around 10^{-10} M .

Of the four mutants designed in this study, Avm-Y33H turned out to be most successful in terms of the reversibility of biotin binding. The idea for this particular substitution came from Nature, since two of the sea urchin fibropellins contain a histidine residue at this position in their avidin-like domain [29,30]. Interestingly, this avidin variant exhibited a clear pH dependence on its binding to biotin-derivatized surface. At neutral pH (or lower), Avm-Y33H bound irreversibly to this surface, but at elevated pH (pH 9 or higher) about 50% of this protein could be released with free biotin (Figure 2). Interestingly, under the same conditions, chemically modified nitro-avidin [14], used as a positive control, exhibited approx. 65% reversibility, indicating a high level of reversibility for Avm-Y33H. The most likely explanation for the behaviour of Avm-Y33H is that, at lower pH, the histidine residue is positively charged and can therefore interact with the carbonyl group of the biotin (Figure 5). However, when the pH is raised, the histidine loses its proton, the interaction with biotin is lost and binding is partly reversed; this is similar to the situation observed for nitro-avidin [14]. On the other hand, introducing an uncompensated charge into an enclosed site, such as the biotin-binding site of avidin, is energetically unfavourable, suggesting that the affinity of Avm-Y33H to biotin might also be

slightly reduced at lower pH. However, we were unable to verify this reduction, since Avm-Y33H displayed virtually irreversible binding to the biotin surface at pH 7. With 2-iminobiotin the situation is different, however, since at low pH the guanido group of 2-iminobiotin is also positively charged, causing repulsion with the positively charged histidine residue and hence no binding. At higher pH, both the guanido group of 2-iminobiotin and the histidine residue lose the positive charge and the repulsion ceases.

When the binding of Avm-Y33F, Avm-Y33A and Avm-Y33H to 2-iminobiotin was measured (Table 1) only the off-rate was significantly affected by the mutations when compared with that of the wild-type avidin. On the other hand, the on-rates of these four proteins were similar, suggesting that these substitutions do not seem to generate major changes in the shape of the binding pocket. In this regard, it is interesting to note that one of the major reasons for the extremely high affinity of avidin for biotin has been suggested to be shape complementary, i.e., the ligand binding pocket of avidin appears to be precisely designed to fit biotin; therefore conformational changes requiring energy are not needed upon binding [31]. In the case of Avm-Y33Q, the situation seemed to be totally different, since this mutant displayed substantially slower binding to biotin than that observed for wild-type avidin, indicating that the introduction of the longer side chain of glutamine to the binding site may somehow distort the delicate shape of the binding pocket.

Another interesting aspect of these tyrosine substitutions was that the tetrameric structures of Avm-Y33A, Avm-Y33Q and Avm-Y33H were clearly less stable than those of the wild-type avidin or Avm-Y33F (Figure 2). In the absence of biotin, these proteins dissociated partly or fully into monomers already at room temperature when SDS was present. However, in non-denaturing conditions, all the mutants migrated as tetramers in FPLC gel-filtration columns, similarly to the behaviour of wild-type avidin. Furthermore, binding to biotin also increased the stability of these three variants, but they still displayed substantially weaker subunit association than those observed for wild-type avidin or Avm-Y33F. It seems that although Tyr-33 is situated deep in the avidin barrel, relatively distant from the monomer-monomer interfaces, its replacement can cause significant disturbances in the inter-monomer interactions, leading to impaired quaternary structure. A similar decline in stability was also detected with nitro-avidin [14]. Moreover, in a recent communication, Qureshi et al. [23] reported that in streptavidin substitution of three residues (Ser-45, Thr-90 and Asp-128) that

interact with biotin led to the formation of completely monomeric protein. Taken together, these results provide further evidence for our earlier suggestion that the high-affinity binding and the remarkable stability of avidin (and streptavidin) are interrelated, and it is difficult to modify either one without affecting the other. In this regard, additional structural studies of these tyrosine mutants together with structural data from our previously published dimeric/monomeric avidin variants [9,32] may provide further insight into the formation and stability of the avidin tetramer. In conclusion, we have shown clearly in this study that the elimination of H-bonds does not necessarily reduce the binding or the affinity of avidin to biotin, as long as the shape or the complementarity of the binding is preserved.

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