Reversibility of biotin-binding by selective modification of tyrosine in avidin

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The tight interaction between the vitamin biotin and the protein avidin is so strong ($K_a \sim 10^{15}$ M^{-1}) that conditions which are usually sufficient for protein denaturation fail to dissociate the avidin–biotin complex. In order to form a reversible interaction between the two biomolecules, we have modified the binding-site tyrosine by nitration, thus reducing the pK_a of the phenol group which forms a crucial hydrogen bond with the ureido group of biotin. At relatively low pH values (4–5), the resultant modified forms of avidin bind biotin with a very high association constant ($> 10^9$ M^{-1}). The modified avidins are thus capable of supporting stable, long-term binding of biotin or biotinylated macromolecules. The latter molecules can be detached by increasing the pH of the medium or by introduction of excess levels of biotin at neutral pH. These findings demonstrate the importance of a single hydrogen bond for strong biotin binding. The new derivatives of avidin should be useful for applications whereby a reversible interaction between the four biotin-binding sites and biotin is desired, thus increasing the versatility of the avidin–biotin system for biotechnological application.

INTRODUCTION

The complex formed between the vitamin biotin and the egg-white glycoprotein avidin (or the related bacterial protein streptavidin) is the strongest interaction known between a ligand and a protein [1]. This strong interaction has been utilized for a large number of biotechnological applications [2–4]. The solution of the crystal structures for both avidin [5,6] and streptavidin [7,8] and their interaction with biotin has provided a tool for the better understanding of protein–ligand interactions in general. Owing to the availability of such information, studies on site-directed mutagenesis and chemical modifications of such proteins can be planned in a more intelligent manner.

The three-dimensional structures of avidin and streptavidin and their respective complexes with biotin have suggested that one of the most important bonds in the binding of biotin is the hydrogen bond formed between the carbonyl group on the ureido ring of biotin and the single tyrosine (Tyr-33) in avidin (or the analogous tyrosine, Tyr-43, in streptavidin). These findings confirmed the chemical modification studies [9], which showed that alkylation of the phenolic moiety with *p*-nitrobenzene-sulphonyl fluoride results in the total inactivation of biotin binding by both proteins. Analysis of the crystal structures of avidin and streptavidin also suggest that there is room in the binding pocket to accommodate modification of the *ortho* position of the phenol in tyrosine without obstructing biotin binding. It is therefore not surprising that the binding activity of radioiodinated avidin was at least partially retained [10].

In the present study, the binding-site tyrosine residue in avidin and streptavidin was modified chemically at the *ortho* position by nitration and iodination. We demonstrate that such electrophilic modification does not result in the elimination of biotin-binding activity; rather, the binding by the modified protein is restricted to lower pH values, due to the reduction of the pK_a of the phenol. The modified forms of avidin and streptavidin are thus reversible in their biotin-binding properties, thereby offering new possibilities in their application in avidin–biotin technology. The present paper also demonstrates the importance of the hydrogen bond for biotin binding and its controlled perturbation by selectively modifying the *ortho* position of the binding-site tyrosine residue. These chemically modified proteins provide an easy and attractive alternative to site-directed mutagenesis to produce reversible and hybrid forms of avidin or streptavidin.

EXPERIMENTAL

Materials

Avidin was obtained from Belovo Chemicals (Bastogne, Belgium) or from STC Laboratories (Winnipeg, Manitoba, Canada). Streptavidin was prepared according to a previously published procedure [11]. Tetranitromethane was a product of Fluka AG (Buchs, Switzerland). D-[*carbonyl*-^{14}C]Biotin (57 mCi/mmol; [^{14}C]biotin), was obtained from Amersham International (Amersham, Bucks., U.K.). Sepharose 4B-CL was purchased from Pharmacia (Uppsala, Sweden). Avidin was immobilized on Sepharose by the CNBr procedure according to the method of Wilchek and co-workers [12,13], using 2 mg of protein per g of resin.

Citrate/phosphate buffers (50 mM) were used between pH 3 and pH 6, 50 mM Tris/HCl buffers between pH 7 and pH 9, and 50 mM sodium carbonate/HCl buffer was used for pH 10.

Preparation of nitro-avidin

Nitro-avidin was prepared according to the published procedure [14]. Samples (5 mg in 1 ml of 50 mM Tris buffer, pH 8) of avidin were treated with different concentrations (1.6–40 mM) of tetranitromethane for 30 min at 23 °C. The samples were dialysed overnight: once against 4 litres of 1 M NaCl and twice against double-distilled water. The amount of modified tyrosine in the sample was determined spectrophotometrically ($\epsilon_{428} = 4200$ $M \cdot cm^{-1}$) and by amino acid analysis.

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Streptavidin (2.5 mg per ml of buffer) was subjected to a similar nitration procedure using higher levels (50 mM) of tetranitromethane.

The modified forms of the various proteins were examined by FPLC using a Superose 12 HR 10/30 column (Pharmacia).

Preparation of iodo-avidin

An aliquot (10 μl) of KI (32 mg/ml) was added to a solution of avidin (2 mg in 0.5 ml of 0.5 M sodium phosphate buffer, pH 7). Chloramine T (0.2 ml of a 2 mg/ml stock solution) was added, and the reaction was allowed to proceed for 30 min at 23 °C. Sodium *m*-bisulphite (0.3 ml of a 2 mg/ml stock solution) was added, and after 1 min a second aliquot (1 ml) of 1 % (w/v) KI was added. The iodo-avidin was then dialysed overnight against double-distilled water.

Preparation of nitro-avidin affinity columns

Columns of nitro-avidin were prepared by two different strategies. One approach involved the direct immobilization of purified nitro-avidin on Sepharose by the CNBr procedure using 0.5 mg of protein per g of resin. Immobilized nitro-streptavidin was prepared by an identical procedure.

In an alternative method, avidin, preimmobilized to Sepharose (1.4 mg of protein per ml of resin), was subjected to modification by tetranitromethane. To a 4-ml suspension of avidin–Sepharose (2 ml of resin in 2 ml of 50 mM Tris buffer, pH 8), 6 μl of the concentrated liquid reagent was added directly to the resin. After a 50-min incubation period at 23 °C, the resin was washed extensively using PBS, pH 7.4. The modified resin was yellow in colour, reflecting the conversion of tyrosine into nitrotyrosine.

Blocking of unmodified biotin-binding sites

Since only partial modification of the binding-site tyrosine could be achieved under the described conditions, unmodified sites could potentially pose a problem in subsequent applications of the nitro-avidin. Thus, following nitration, samples were selectively blocked with excess levels of free biotin using 0.6 mM biotin in a desired buffer (e.g. citrate/phosphate buffer pH 4, although virtually any buffer is acceptable as a blocking buffer). Biotin molecules, which occupy the modified biotin-binding sites, were released using sodium carbonate buffer, pH 10, after which the nitro-avidin preparation was brought to pH 4 or 5 with citrate/phosphate buffer. Biotin molecules which occupy the unmodified biotin-binding sites are retained under these conditions. For solutions of nitro-avidin, these steps were performed by dialysis against the appropriate buffers. In the case of immobilized nitro-avidin, either the biotin-containing buffer was passed through a column or the blocking was performed batchwise by centrifugation.

Binding of biotinylated proteins to nitro-avidin

The binding of biotinylated proteins to nitro-avidin was tested in two ways. In one experiment, nitro-avidin was immobilized to Sepharose as described above, and samples of biotinylated BSA (20 μg) in a desired buffer (100 μl) were applied batchwise to 100 μl of the nitro-avidin–Sepharose resin. Following centrifugation, the supernatant fractions were measured for protein [15]. The percentage of binding at different pH values was determined by subtracting the amount of protein in the effluent fractions from that applied to the resin.

A complementary microtitre plate enzyme assay system was also used to assess the comparative biotin-binding activity of native avidin and nitro-avidin. In this procedure, microtitre plates were coated with the desired protein (1 μg/100 μl of PBS per well), and different concentrations of biotinylated horseradish peroxidase (between 10 ng and 1 μg in 150 μl of citrate/phosphate buffer, pH 4) were added. The plates were washed and the peroxidase activity was determined using 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) as a substrate (2.5 mg per 10 ml of citrate/phosphate buffer, pH 5, to which 10 μl of 30 % hydrogen peroxide was added). Colour formation was measured at 420 nm.

Determination of association constant

Samples of nitro-avidin (25 μg in 50 μl) were mixed with solutions (1–20 μl) of [^{14}C]biotin (final concentration of between 0.5 and 2.5 μM) and brought to a final volume of 200 μl with 50 mM citrate/phosphate buffer. After 48 h of incubation at 4 °C, the solution was mixed with 200 μl of a suspension of SP-Sephadex (20 mg/ml). A BioSpin chromatography column (Bio-Rad Laboratories) was used to separate the resin from the supernatant fluids, and an aliquot of the citrate/phosphate buffer, pH 4 (400 μl), was used to wash any remaining free biotin from the adsorbed nitro-avidin. Radioactivity associated with the free and bound (applied minus free) fractions was determined, and the results were analysed according to Scatchard [16]. A control for free biotin was employed, which consisted of an equivalent aliquot of [^{14}C]biotin in the absence of avidin.

Stability of avidin and streptavidin derivatives

Aliquots (15 μg in 20 μl of sample buffer) of the desired protein were incubated for 30 min at selected temperatures and subjected to SDS/PAGE on 15 % gels (E. A. Bayer, S. Ehrlich-Rogozinski and M. Wilchek, unpublished work). The gels were stained using Coomassie Brilliant Blue. The stability features of the protein molecule were followed by dissociation of the tetramer to the monomeric form. For this purpose, densitometry tracings of the individual lanes were analysed using the Molecular Analyst®/Macintosh image analysis software for the Model GS-670 imaging densitometer (Bio-Rad, Hercules, CA, U.S.A.). The bands associated with the tetramer and monomer in each lane were quantified, and the results (% dissociation) were standardized and plotted as a function of temperature.

Modelling experiments

The probable position of the nitro group in avidin was determined by modelling studies using the co-ordinates deposited in the Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, NY 11973, U.S.A. (reference 2AVI).

RESULTS

Preparation of nitro-avidin

Samples of egg-white avidin, which contains a single tyrosine residue located in the biotin-binding site, were treated with different concentrations of tetranitromethane. The percentage of modified tyrosine in each sample was determined by amino acid analysis. Under the conditions of the modification procedure, tyrosine was the only amino acid which appeared to be modified. Maximum levels (approx. 70 %) of modification were achieved using > 20 mM reagent (Figure 1).

An average of about three out of the four tyrosines of the avidin tetramer thus appeared to be modified by nitration.

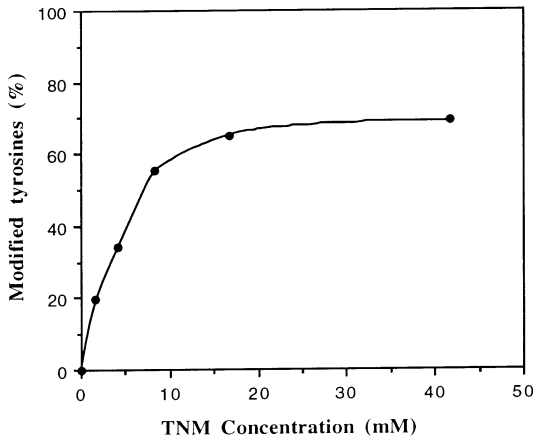


Figure 1 Modification of avidin as a function of tetranitromethane (TNM) concentration

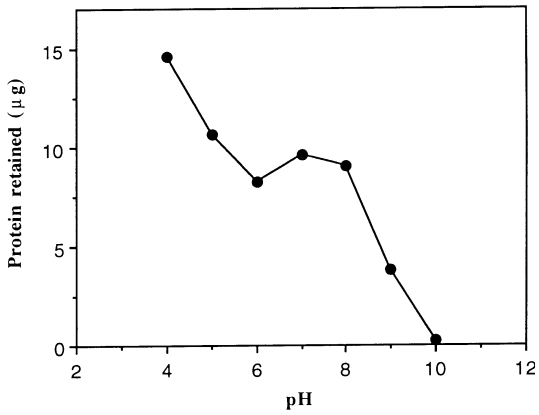


Figure 2 The effect of pH on the binding of biotinylated BSA to a nitro-avidin–Sepharose column

Samples (20 μg) of the biotinylated protein in a buffered solution of the given pH were applied to 0.1 ml of nitro-avidin–Sepharose resin.

Consequently, the modification resulted in hybrid form(s) of avidin in which an average of three monomers contained a nitrotyrosine residue and one of the monomers contained tyrosine in its native form. The preparation itself described herein may be of importance for a variety of biotechnological applications (E. Morag, E. A. Bayer and M. Wilchek, unpublished work).

In order to achieve complete reversibility (for the purposes of the present work), the remaining unmodified sites in the nitro-avidin preparation were blocked selectively as described in the Experimental section.

Streptavidin (which contains six tyrosines per subunit, only one of which is located in the binding site) was also subjected to a similar nitration procedure. In this case, higher levels of tetranitromethane were used, owing to the higher number of tyrosines per subunit.

Properties of nitro-avidin

The effect of pH on the binding of biotinylated proteins to nitro-avidin was determined by the interaction of biotinylated BSA with an avidin–Sepharose column. Optimal binding was found

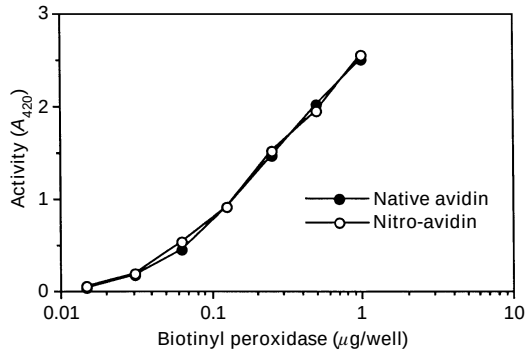


Figure 3 Binding of biotinylated peroxidase to nitro-avidin and native (unmodified) avidin

The indicated amount of biotinyl enzyme was added to microtitre wells coated with the desired avidin sample, and enzyme activity was measured colorimetrically (A_{420}). Under the conditions of the experiment, the capacity for biotin binding of the modified and unmodified avidin was identical.

to occur at pH 4 (Figure 2). At higher values of pH (between 5 and 8), plateau levels of binding were observed. Above pH 8, the binding dropped markedly and at pH 10 was negligible. Similar results were achieved using biotinylated peroxidase in a microtitre-plate enzyme assay (results not shown). Under optimal conditions (at pH 4), the binding activities of nitro-avidin and native (unmodified) avidin were essentially indistinguishable (Figure 3).

Attempts to calculate the affinity constant (K_a) between nitro-avidin and biotin were carried out at pH 4 by a modification of the original method published by Green [17]. The results revealed that below saturating concentrations of biotin, nearly all of the vitamin was associated with avidin. The results were plotted according to Scatchard [16], and a value greater than 10^9 M^{-1} was achieved. The difficulties in obtaining precise measurements for such high-affinity constants have been discussed by Green in his original publications on avidin and subsequent reviews on the subject [1,17,18].

Biotin or biotinylated molecules could be completely released at pH 10 (Figure 4). Alternatively, excess concentrations of

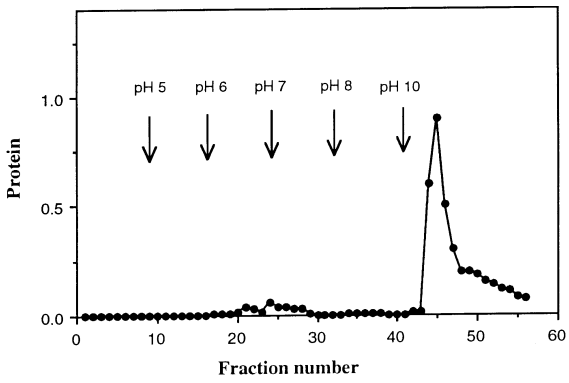


Figure 4 Release of biotinylated BSA from a nitro-avidin–Sepharose column as a function of pH

A sample containing 1.5 mg of biotinylated BSA was applied to a 2-ml nitro-avidin–Sepharose column, and the column was washed with the appropriate buffers at the indicated pH. Fractions of 1 ml were collected, and protein content (A_{600}) was determined by the Bradford method [15].

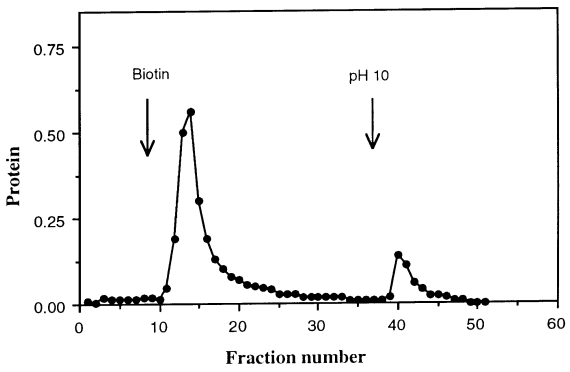


Figure 5 Biotin-mediated release of biotinylated BSA from a nitro-avidin–Sepharose column

A sample containing 1.5 mg of biotinylated BSA was applied to a 2-ml nitro-avidin–Sepharose column. The column was washed with a solution containing 0.6 mM biotin in 50 mM citrate/phosphate buffer, pH 4. Residual biotinylated BSA was eluted from the column with 50 mM carbonate buffer, pH 10. Fractions of 1 ml were collected and protein content (A_{600}) was determined by the Bradford method [15].

biotin (0.6 mM) at any pH between 4 and 10 could be used to displace biotinylated material from avidin (Figure 5). The binding and release of biotin by nitro-avidin could be repeated continuously with no observable reduction in its performance.

Gel filtration of modified forms of avidin on a Superose 12 column showed a single symmetrical peak, identical to the position of unmodified avidin (results not shown). This indicates that the tetrameric structure of the modified protein in solution is retained.

The stability of the nitro-avidin tetramer was compared with the native unmodified protein by an SDS/PAGE assay (E. A. Bayer, S. Ehrlich-Rogozinski and M. Wilchek, unpublished work). Samples (7 μg) were incubated for 20 min at the designated temperature (25 °C, 40 °C, 55 °C, 70 °C and 100 °C) in the presence of SDS-containing sample buffer, and the dissociation of the tetrameric to the monomeric form was observed (Figure 6). Note that at relatively low temperatures, avidin appears to aggregate, and the aggregates fail to penetrate the interface between the stacking and separating gels. This aggregation is presumably caused by the interaction of the negatively charged detergent with the positively charged tetrameric protein. At higher temperatures, the aggregates dissociate, and the protein penetrates the gel mainly as the monomer with residual amounts of dimer.

The ratio of monomer to tetramer (% dissociation) was quantified by analysing the densitometry tracings of the individual lanes and by plotting as a function of temperature (Figure 7). The tetramer–monomer transition of the native (underivatized) form of avidin occurred at about 50 °C. In contrast, near-complete dissociation of the nitro-avidin tetramer was observed at room temperature (in the presence of SDS), indicating a more delicate quaternary structure. The binding of biotin caused a stabilization of both the native and modified forms of avidin. Again, the tetrameric structure of the biotin–nitro-avidin complex was less stable than that of the complex between biotin and the unmodified avidin.

Similar biotin-binding and -release properties were observed for nitro-streptavidin (Figure 8). Nitration of streptavidin also resulted in reduced stability properties of the protein (results not shown).

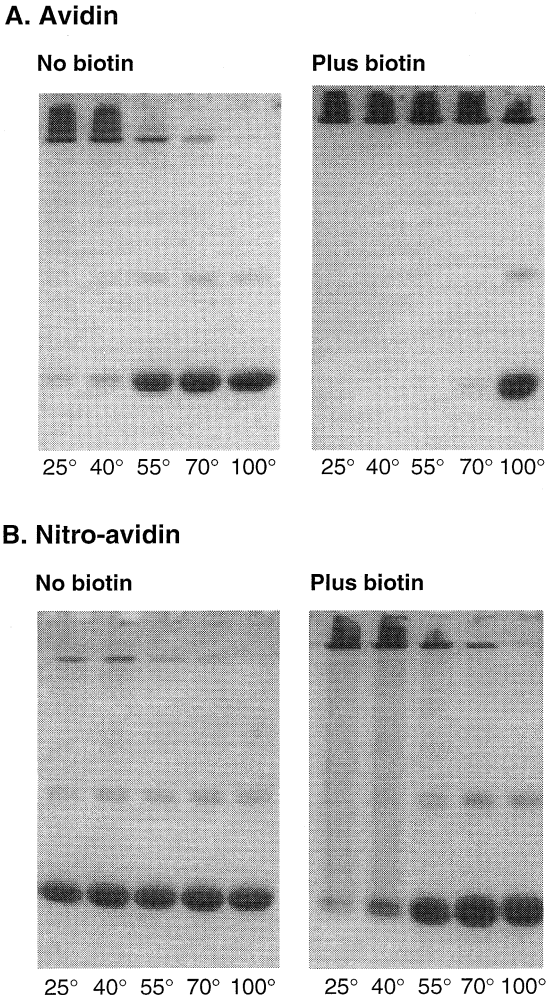


Figure 6 Comparative heat-stability in SDS of avidin and nitro-avidin

Biotin-free or biotin-complexed samples of avidin or nitro-avidin were preincubated for 20 min in the presence of SDS at different temperatures. Aliquots (7 μg) were subjected to SDS/PAGE and the gels were stained with Coomassie Brilliant Blue. The aggregated avidin and nitro-avidin tetramers remained in the stacking gel at lower temperatures, whereas the dissociated monomers and dimers penetrated into the separating gel.

DISCUSSION

Site-directed mutagenesis is currently the preferred approach for modifying a protein and for studying the relationship between its structure and its function. In the case of avidin, the recent solution of its three-dimensional structure [5,6] and its comparison with that of streptavidin [7,8] showed clearly which residues are involved in the binding of biotin. These binding-site residues would be prime targets for site-directed mutagenesis. For many purposes, an ideal modified avidin would be one which retained the biotin-binding function with a similarly high affinity constant, but which could be easily reversed. Site-directed mutagenesis appeared to be a promising tool for such studies. Indeed, recent publications [19,20] describe the exchange of one of the important binding-site residues in streptavidin, wherein Trp-120 was converted into Phe; although the biotin-binding property was retained in the resultant modified protein, its affinity constant was significantly lower than that of the native protein.

We have recently shown the existence of streptavidin analogues

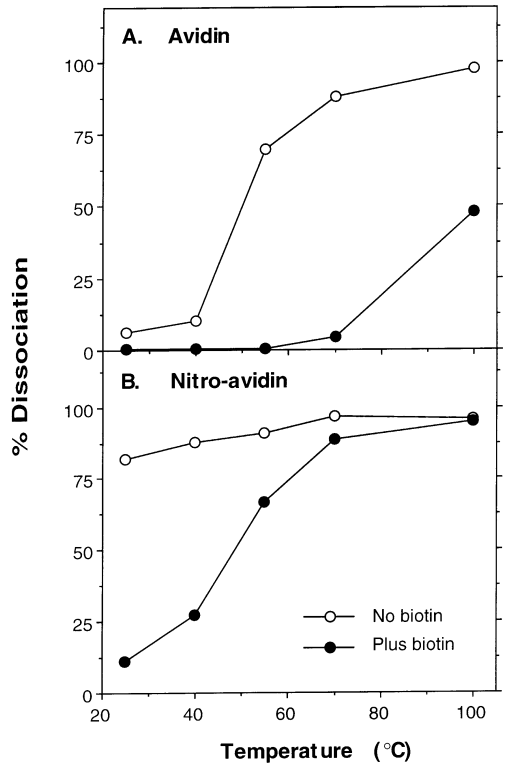


Figure 7 Quantification of the tetramer–monomer transition

The distribution of the various quaternary forms (monomer, dimer and aggregated tetramer) of the avidin samples shown in Figure 6 was analysed by densitometry, and the percentage dissociation of the tetrameric forms to monomer and dimer was determined.

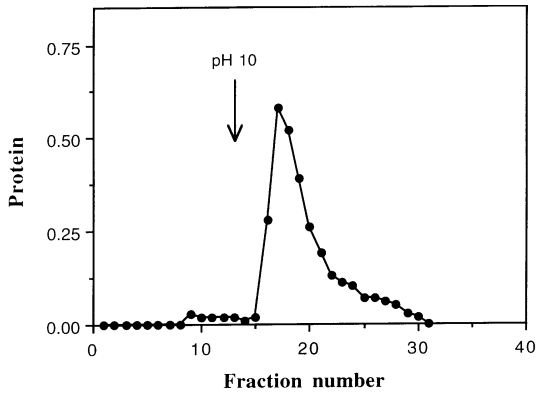


Figure 8 Release of biotinylated BSA from a nitro-streptavidin–Sepharose column as a function of pH

A sample containing 1.5 mg of biotinylated BSA was applied to a 2-ml nitro-streptavidin–Sepharose column, and the column was washed with 50 mM carbonate buffer, pH 10. Fractions of 1 ml were collected and protein (A_{600}) was determined by the Bradford method [15].

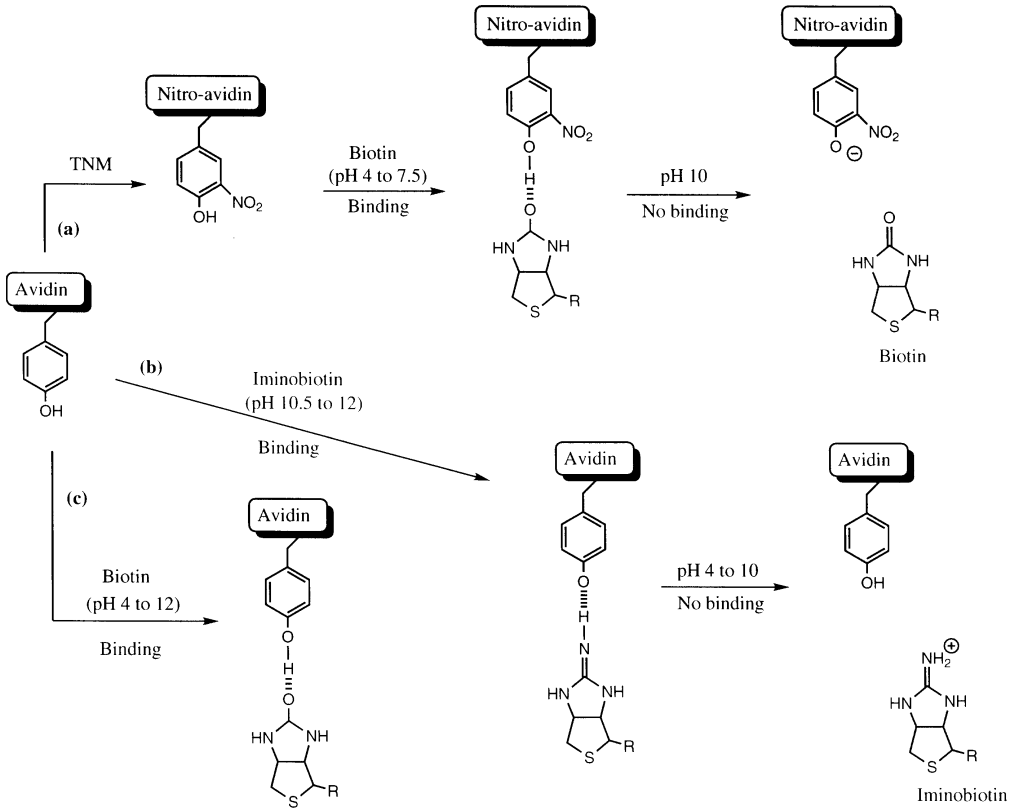
in selected strains of *Streptomyces venezuelae*, thus demonstrating that the biotin-binding protein is not restricted to the single species, i.e. *Streptomyces avidinii* [21]. That study implied that nature is extremely conservative in the formation of the biotin-binding site and that changes in the amino acid residues may simply destroy the binding altogether. Thus, in order to achieve reversible biotin binding with a high affinity constant, one should use the information gained by the three-dimensional studies and perform small and permissible modifications of selected binding-site residues.

The X-ray structure of avidin showed clearly that the single tyrosine of the subunit (Tyr-33) forms a critical hydrogen bond in concert with two other binding-site residues, thus forming an oxyanion with the carbonyl oxygen of biotin. A similar bond is formed by its counterpart (Tyr-43) in streptavidin. Chemical modification of the tyrosine at the hydroxyl group in both proteins eliminated the biotin binding [9]. Thus, in considering the customary conversion of tyrosine into phenylalanine by site-directed mutagenesis, the resultant form of avidin would most probably be inactive.

In the present study, we decided to take an alternative approach, combined with modelling studies. We have shown that the binding behaviour of avidin towards biotin or biotinylated compounds can be dramatically altered by chemical modification of the binding-site tyrosine at the *ortho* position. The modelling experiments have indicated that there is room in the binding site to accommodate only a single nitro group into Tyr-33, without causing extensive distortion of the binding site. Such a modification reduces the pK_a of the hydroxy group of the tyrosine residue, and the binding of biotin is reversible, since at high pH values hydrogen bonds cannot form. Thus, the complex is essentially non-dissociable at low and neutral pH levels, but easily dissociable either at higher pH (i.e. > 10) or in the presence of excess concentrations of biotin at any pH. It is interesting to note that such a reversible system was recently described for an antibody–hapten complex [22]. In this case a nitro group was introduced in the binding site of an antibody. The hapten bound to the antibody at low pH, but was released at higher pH values.

The nitration of avidin reduces the pK_a of the tyrosine residue. The hydrogen bond formed between the hydroxyl group of the tyrosine and the carbonyl group of biotin is thus perturbed at pH values higher than the pK_a of nitrotyrosine, and this single change has a profound effect on the binding of biotin (Scheme 1). This finding emphasizes the importance of this single hydrogen bond in biotin binding. It also explains several other features of the avidin–biotin system. For example, it has been shown that iminobiotin binds to avidin only at pH values higher than 10.5 [23,24]. This finding was explained as the neutralization of the guanidine group of iminobiotin which makes it equivalent to the carbonyl oxygen of biotin. The results with nitrotyrosine show clearly that this is a type of hydrogen bond interplay; up to pH 10.5 (the pK_a of tyrosine), the tyrosine contributes the hydrogen for hydrogen bond formation, which is required for biotin binding. Iminobiotin binds at higher pH because, under these conditions, the guanido group can contribute the required hydrogen to form the hydrogen bond (see Scheme 1b). At lower pH values, the hydrogen bond cannot be formed between the ionized guanido group and the hydroxy group of tyrosine, and, consequently, there is no binding.

According to this scenario, the avidin–biotin complex should be dissociated at higher pH values, but this does not happen below pH 13. The reason for this may be that, once the hydrogen bond is formed, it is difficult to dissociate the bond, since it is completely shielded from the environment. Another possibility is that the tyrosine may have an unusually high pK_a, as do buried tyrosines in other proteins [25]. Indeed, we have shown previously that the tyrosine in avidin is buried [26], and, when biotin occupies the binding pocket, it is unavailable for interaction with small molecules such as acrylamide and iodine. Thus, conditions of very high pH are required to dissociate the avidin–biotin complex.



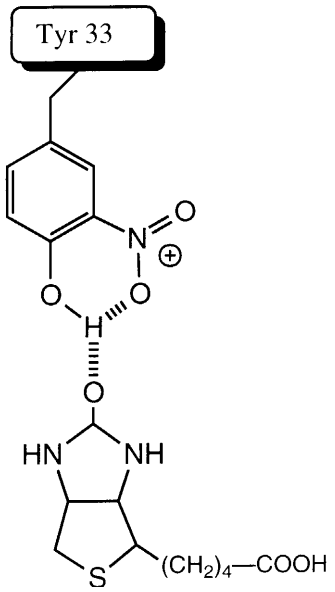
Scheme 1 Overall scheme showing the interaction of biotin and iminobiotin with either unmodified avidin or avidin modified at the *ortho* position of the binding-site tyrosine residue

In (**a**), treatment of avidin with tetranitromethane (TNM) yields nitro-avidin, which binds biotin at low and neutral pH but fails to bind at high pH. In contrast, as shown in (**b**), iminobiotin binds to unmodified avidin under conditions of high pH, but fails to do so at low and neutral pH. In (**c**), the native avidin molecule binds to biotin at low, neutral and high pH.

It is therefore not surprising that the complete dissociation between nitro-avidin and biotin takes place only at pH 10, even though the pK_a of nitrotyrosine is generally around 7.2. The pK_a of nitrotyrosine in nitro-avidin may in fact be higher due to its buried position and hydrophobic environment. Yet another possibility is that the phenolic hydrogen of the nitrotyrosine may participate in two hydrogen bonds: an internal hydrogen bond with the nitro group and the bond with the carbonyl oxygen of biotin which stabilizes the complex up to pH 10 (Scheme 2).

Another interesting characteristic of nitro-avidin and nitro-streptavidin is that the tetrameric structures of both modified proteins are clearly less stable than those of the respective parent molecules. Although the binding to biotin increases the stability of the modified avidin, it is still less stable than the complex formed between biotin and native avidin. This indicates that the presence of the nitro group in the binding site of avidin serves to perturb the inter-monomer interactions which are responsible for tetramer formation, despite the fact that the presumed position of the nitro group is relatively distant from those interactions. Additional structural studies and preparation of mixed hybrids between native and nitro-avidin subunits may provide further insight into the effect of modified tyrosine on the stability of the tetramer.

On the practical side, the nitro-avidin derivative described in this paper may provide the long-anticipated universal avidin (and streptavidin) column [27]; namely, a column which not only possesses four stable, high-affinity, biotin-binding sites for biotin, but is completely reversible under mild conditions. Thus, reasonable conditions of pH or competition with free biotin will remove



Scheme 2 Alternative scheme for hydrogen bonding between nitro-tyrosine and biotin

the biotinylated component unscathed from the modified avidin column. In this manner, the immobilized avidin (in this case nitro-avidin) can be regenerated for subsequent use. Preliminary

results have shown that iodo-avidin (i.e. a modified form of avidin in which the binding-site tyrosine was converted into iodo-tyrosine at the *ortho* position) also behaves in a similarly reversible manner with respect to its binding of biotin. Such modified derivatives of avidin and streptavidin should be applicable for all applications of avidin–biotin technology, wherein both reversibility and the interaction of the four intact binding sites are advantageous. In this context, affinity-based separations in which immobilized avidin serves as a capture system may be considered in which precious biotinylated molecules (e.g. receptor components, hormones, etc.) are employed or sensitive biological target materials (e.g. cell subpopulations) are desired. Experiments in this direction are currently in progress.

Finally, we have shown in this paper that chemical modification may be easier to apply in certain cases where introduction of natural or unusual amino acids by site-directed mutagenesis would be unreasonable.

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