

Limited proteolysis of native proteins: The interaction between avidin and proteinase K



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Abstract

Avidin is a tetramer of 16-kDa subunits that have a high affinity for biotin. Proteolysis of native apoavidin by proteinase K results in a limited attack at the loop between β -strands 3 and 4, involving amino acids 38–43. Specifically, sites of proteolysis are at Thr 40–Ser 41 and Asn 42–Glu 43. The limited proteolysis results in an avidin product that remains otherwise intact and which has enhanced binding for 4'-hydroxyazobenzene-2-benzoic acid (HABA), a chromogenic reporter that can occupy the biotin-binding site. Saturation of the biotin-binding site with the natural ligand protects avidin from proteolysis, but saturation with HABA enhances the rate of proteolysis of the same site.

Analysis of the three-dimensional structures of apoavidin and holoavidin reveals that the 3–4 loop is accessible to solvent and scores highly in an algorithm developed to identify sites of proteolytic attack. The structure of holoavidin is almost identical to the apoprotein. In particular, the 3–4 loop has the same structure in the apo and holo forms, yet there are marked differences in proteolytic susceptibility of this region. Evidence suggests that the 3–4 loop is rather mobile and flexible in the apoprotein, and that it becomes constrained upon ligand binding. In one crystal structure of the apoprotein, this loop appears constrained by contacts with symmetry-related molecules. Structural analyses suggest that the “lid” to the biotin-binding site, formed by the 3–4 loop, is displaced and made more accessible by HABA binding, thereby enhancing its proteolytic susceptibility.

Keywords: avidin; limited proteolysis; predictive algorithm; proteinase K

Many physiological events are mediated by limited proteolytic attack upon a native protein structure (Bond & Beynon, 1987). Further, limited proteolysis has been used to generate derivatives of native proteins that retain some biological or structural properties (Price & Johnson, 1989). However, the molecular recognition events that are involved in the interaction between the proteinase and the native protein substrate are poorly understood. For example, although the majority of Lys-X and Arg-X bonds in any protein will be located on the surface of a protein, and presumably accessible to the action of trypsin, it is a common observation that only some, one, or even none of these bonds are cleaved. It might be imagined that proteolytic sites adopt a conformation that matches the substrate when it is bound to the enzyme, minimizing the loss of conformational entropy upon binding. For example, the conserved structure of the

binding loop of the small serine proteinase inhibitors (Bode & Huber, 1992) might be expected to provide a structural paradigm for proteolytic nick sites. Equally plausible, however, is the possibility that the proteolyzed loop is flexible and that the favorable binding energies can overcome the loss of conformational entropy that is attendant on binding. In this instance, relatively weak interactions of a nick-site loop with the rest of the protein structure must allow the loop to break free and adopt a substrate-like conformation. There is scope for a degree of “induced fit” in this process, whereby a limited substrate:proteinase interaction may process along the enzyme subsites, enhancing binding as each subsite is filled. The ability of the loop to break away from the protein core, the flexibility of the loop once bound to the enzyme at one or two subsites, and the strength of the interactions between the subsites and the amino acid side chains in the loop provide obstacles to productive interaction between substrate and enzyme. Such obstacles may explain the wide range of kinetic constants that can be measured for limited proteolytic reactions.

There have been few studies on macromolecular interactions in limited proteolysis. Early attempts to discover the underlying

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ing structural criteria focused on correlations with segmental mobility (Vita et al., 1988; Fontana, 1989) and accessibility (Novotny & Brucoleri, 1986) and concluded that nick sites are typically found in exposed, flexible loops. A more recent attempt to correlate the structural features of nick sites with those of proteinase inhibitors revealed that nick sites do not, in general, possess a "substrate-like" conformation (Hubbard et al., 1991, 1994). Furthermore, substantial conformational change, local to the scissile bond, would be required if the nick-site region was to adopt the structure exemplified by bound inhibitors. Such observations reinforce the role of segmental mobility in exposure of nick sites.

Avidin is a basic, tetrameric protein (Heney & Orr, 1981) that can bind up to four molecules of biotin with high affinity (Green, 1975) (Kinemage 1). The structure of apo (Livnah et al., 1993a; Pugliese et al., 1994) and holo (Livnah et al., 1993a; Pugliese et al., 1993) forms of avidin are known at high resolution (Kinemage 2). Biotin binding does not apparently introduce major changes in the structure of the subunit (Livnah et al., 1993a; Pugliese et al., 1994), although there are observable changes in physicochemical properties such as thermal stability and intrinsic tryptophan fluorescence. As part of a study of complex proteolytic systems, assembled as biotinylated proteinases on avidin, we noted that avidin is refractory to proteolysis. However, proteinase K was able to effect a limited proteolysis of avidin. We report here the nature of the proteolytic attack and the interrelationship between proteolysis and the ligand-binding site. The data are interpreted in the context of the structure of the apo and holo forms of avidin.

Results

Limited proteolysis of avidin

Avidin has previously been reported to be refractory to proteolysis, and a study designed to generate functionally active derivatives of avidin by limited digestion was unsuccessful (Hiller

et al., 1991). However, the ratios of substrate:proteinase (50:1 by weight) were more typical of proteolysis experiments using denatured substrates, and proteolysis of native proteins can require substantially greater amounts of proteinase (Price & Johnson, 1989).

When avidin (120 μ M monomers, 2 mg/mL) was incubated with proteinase K (30 μ M, 850 μ g/mL) for 1 h and the product analyzed by reducing SDS-PAGE, the 16-kDa avidin band was lost coincidental to the appearance of a broad band at approximately 8 kDa that gave the appearance of being two merged bands of similar size (Fig. 1). A small residue of the material at 16 kDa remained on the gel and could not be digested, even under extended incubation. As will become clear, this was attributable to the small amount of holoavidin in this preparation, most of the material being apoavidin. On nonreducing SDS-PAGE, the 16-kDa avidin band remained intact throughout the proteolytic reaction. Thus, the limited proteolytic attack is directed to the region of the sequence between Cys 4 and Cys 83, the origin of the intramolecular disulfide bond.

The digestion products were separated on reducing SDS-PAGE and blotted onto PVDF membrane for automated Edman degradation. Undigested avidin sequenced correctly, to yield the N-terminus ARKXS-. The proteinase K digestion product yielded a mixture of sequences at different yields: the sequence ARKXS- (110 nmol) representing the avidin N-terminus, and two other sequences at different yields—EIKEXPH- (29 nmol initial yield, back extrapolated through four or five cycles) and SNEIKEXP- (19 nmol). These sequences were unambiguous and consistent with two sites of proteolysis at Asn 42–Glu 43 and Thr 40–Ser 41, respectively, in close proximity in the primary sequence and thus, three-dimensional structure (Kinemage 2). Approximately 60% of the product had the sequence EIK- and 40% the sequence SNEIK-. Proteinase K would not be expected to act very efficiently as a dipeptidase, and it is more likely that the enzyme attacked at two different sites, hydrolyzing Asn 42–Glu 43 slightly faster than Thr 40–Ser 41. Both of these sites are located in the exposed loop between β -sheet strands 3 and 4, which forms a "lid" over the biotin-binding site (Pugliese et al.,

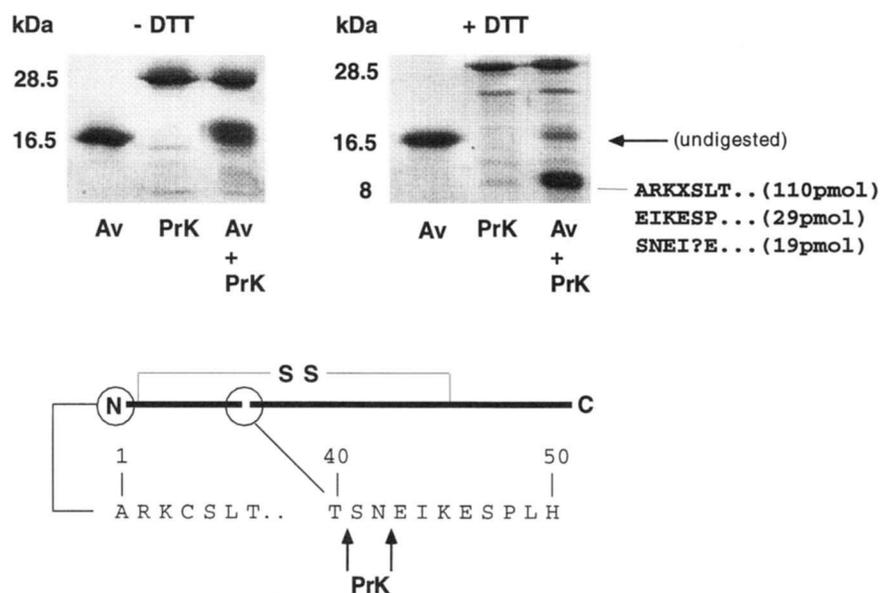


Fig. 1. Limited proteolysis of apoavidin by proteinase K. Avidin (120 μ M monomer) was incubated with proteinase K (30 μ M) for 120 min in 200 mM Tris/HCl, containing 5 mM CaCl₂, pH 8.0. At the end of the digestion period, the reaction was stopped by precipitation of the reaction mixture with 10% (w/v) trichloroacetic acid. After centrifugation, the precipitated proteins were freed of denaturant by repeated washing with ether and run on SDS-PAGE in the presence (+DTT) or absence (-DTT) of reducing agent. The bands indicated were also blotted onto PVDF membrane and subjected to N-terminal sequencing. The sequencing results are included on the diagram.

1994). It has not been possible to clarify the kinetic relationship between the two sites of proteolysis because the rates of digestion are so similar, but it is likely that they are proteolyzed in an exclusive, "either-or" fashion.

Because of the lack of mobility change on nonreducing SDS-PAGE, it is unlikely that there has been further digestion or terminal fragmentation of the two products generated after the primary site of attack. Thus, proteinase K effects a very limited attack on avidin, targeted at one region of the structure. Even under conditions of extended incubation, the reaction does not proceed any further, and the products of the first cleavage are at least an order of magnitude more resistant to further attack. The product of proteinase K attack on avidin was used without further treatment and is referred to as Av^{PrK} .

Effect of proteolysis on ligand binding

The modified avidin was assessed for the capacity to bind 2-(4'-hydroxyphenylazo)benzoic acid (HABA), a chromogenic reporter that binds to the biotin-binding site of avidin and which undergoes a dramatic spectral enhancement at 500 nm. Avidin and Av^{PrK} were titrated with increasing additions of HABA (Fig. 2A) and the A_{500nm} was monitored. The data were corrected for the slight volume expansion, and the corrected data were analyzed by nonlinear curve fitting of the quadratic equation defining noncooperative ligand binding. The values for the dissociation constant were $2.5 \mu M$ and $0.14 \mu M$ for avidin and Av^{PrK} , respectively. Proteolysis therefore resulted in a substantial increase in affinity for this ligand.

In a separate experiment, in which a larger excess of HABA was used, the data were subject to nonlinear curve fitting to derive the extinction coefficients of the free and bound HABA under the conditions of pH and buffer species/concentration used here (results not shown). Free HABA has an extinction coefficient of 900, higher than that reported previously under different buffer conditions. The avidin:HABA complex has a molar extinction coefficient of 28,500, and the Av^{PrK} :HABA complex has a molar extinction coefficient of 35,000. Thus, the environment of the ligand, when bound to the proteolyzed protein, allows a further enhancement of spectral properties. Further evidence for the change in spectral properties is derived from the differences in the slopes of the lines when the Av :HABA and Av^{PrK} :HABA complexes were dissociated by displacement of the chromophore by the tight-binding natural ligand, biotin. However, biotin binds so tightly under these conditions that it was not possible to discern any changes in biotin affinity in Av^{PrK} (Fig. 2B).

When avidin was incubated with HABA and then treated with proteinase K, the absorbance increased in a time-dependent manner (Fig. 3). The increase in absorbance was not evident when proteinase K was incubated alone with HABA, nor when avidin was incubated in the absence of proteinase K. The time-dependent increase in absorbance was fitted as a single exponential, optimizing values for initial and final absorbance and the first-order rate constant. The rate constant was proportional to the amount of enzyme up to about $5 \mu M$ (inset to Fig. 3) and independent of the amount of avidin (not shown), as would be expected for a simple pseudo first-order reaction. Above $5 \mu M$, the proportionality declines, but these digestions are at equimolar avidin and proteinase K, which is atypical for proteolysis studies. It is possible that other factors, such as the rate of unfolding of the 3-4 loop, start to influence the kinetics under these atypical conditions.

Proteolysis of avidin, as monitored by enhancement of HABA binding, yielded a second-order rate constant of $0.032 \text{ min}^{-1} \cdot \mu M^{-1}$, calculated from the data at $5 \mu M$ proteinase K. The ratio of the curve-fitted final and initial absorbance values was 1.225, which is completely consistent with the ratio of 1.228 obtained from the extinction coefficients fitted from the HABA-binding data (Fig. 2A).

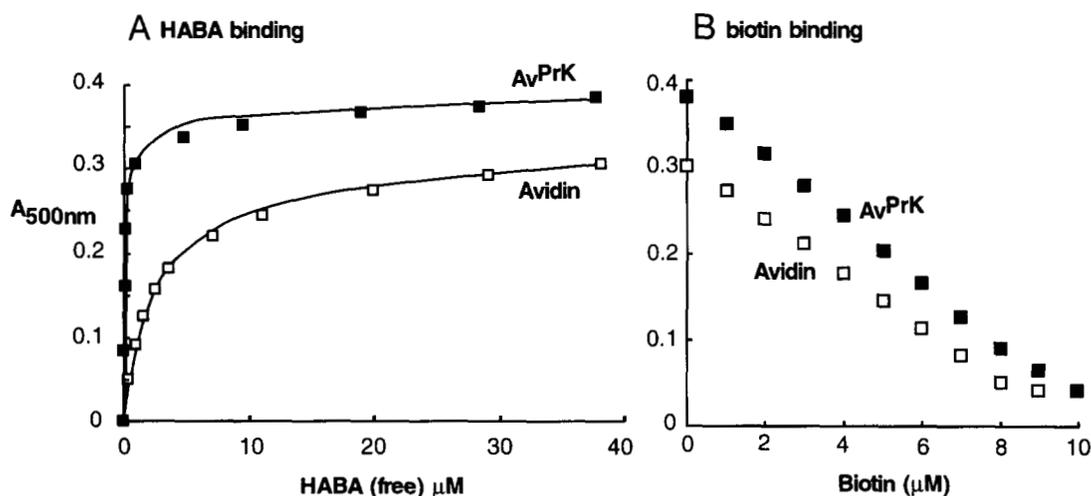


Fig. 2. Ligand binding of native and proteolyzed avidin. Avidin ($120 \mu M$ monomer) was digested with proteinase K ($30 \mu M$) for 240 min. At the end of this time, the proteolyzed avidin was diluted to $12 \mu M$ monomer and the ability to bind HABA (A) was assayed by addition of small volumes of a concentrated solution of the dye, and the increase in absorbance was measured at 500 nm. Subsequently, biotin was added and the A_{500nm} , reflecting displacement of the HABA from the proteolyzed avidin, was recorded (B). Curve fitting of the experimental HABA-binding data was as described in the Materials and methods, and the data are shown as theoretical fitted lines superimposed onto the experimental data.

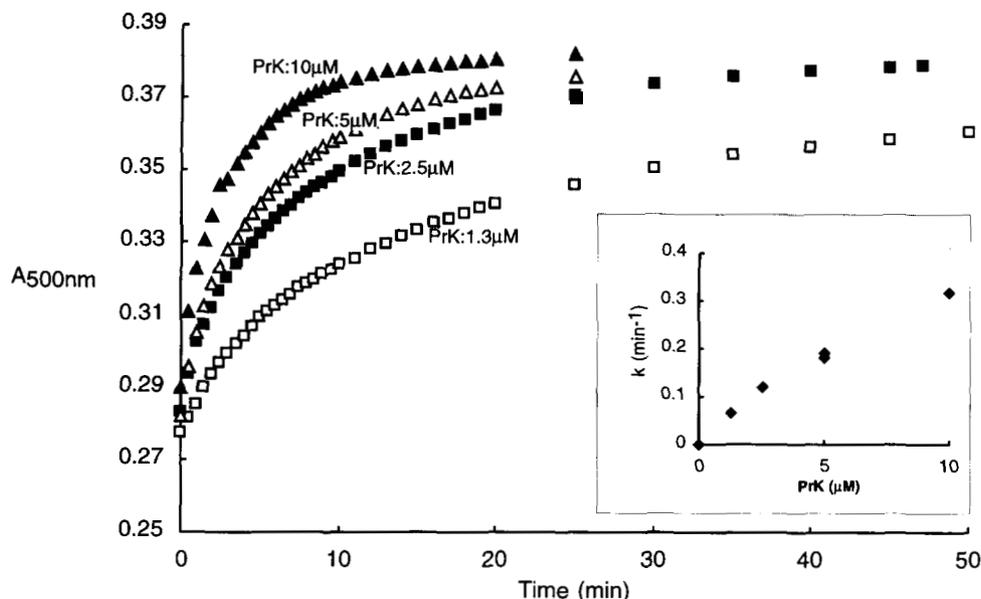


Fig. 3. Enhancement of HABA binding during proteolysis. Avidin ($11 \mu\text{M}$) was preincubated with 0.1 mM HABA before the addition of proteinase K between 0 and $10 \mu\text{M}$ in separate incubations. $A_{500\text{nm}}$ was monitored for up to the next 60 min . Data were analyzed as a single exponential process, optimizing three parameters: initial absorbance, final absorbance, and first-order rate constant. Fitted curves match the data closely and are omitted for clarity. **Inset** graph demonstrates dependence of the first-order rate constant on the concentration of proteinase K.

Effect of biotin on proteolysis

Biotin protects avidin completely from proteinase K attack. Avidin was incubated with proteinase K in the presence or absence of biotin, and samples of the digestion mixture were removed at time intervals and resolved on SDS-PAGE; the stained gel was then scanned by laser densitometry (Fig. 4). The band intensity/time data were analyzed by nonlinear curve fitting to yield a second-order rate constant of $0.002 \text{ min}^{-1} \cdot \mu\text{M}^{-1}$. However, the SDS-PAGE analysis indicated that a proportion of the avidin was resistant to proteolysis and remained undigested even after several hours of incubation. The data could not therefore be described to a simple model in which all of the avidin was digested, but were analyzed in terms of an indigestible residual avidin species. In this preparation of avidin, the amount of this residue as analyzed by densitometry was 12% , which coincided with the proportion of the avidin that was already saturated with biotin on purification (13% , measured by HABA displacement assay, results not shown). When the proteolytic reaction included saturating concentrations of biotin, avidin was completely protected and no digestion was apparent over a 3-h incubation.

Effect of HABA on proteolysis

The protection by biotin is in marked contrast to the effect of HABA, which caused accelerated proteinase K attack on avidin. SDS-PAGE analysis of proteolysis in the presence of HABA showed that the same products were obtained, but at markedly enhanced rates. The concentration of HABA was $100 \mu\text{M}$, and the K_d for native avidin was $2.5 \mu\text{M}$; for Av^{PrK} the K_d was $0.14 \mu\text{M}$. Under such conditions, HABA was present at near-saturation levels. However, when the binding site was fully occupied by HABA, avidin was proteolyzed at a much higher rate (Fig. 5). The second-order rate constant for the digestion in the presence of HABA was $0.025 \text{ min}^{-1} \cdot \mu\text{M}^{-1}$, approximately 10-fold greater than in the absence of ligand, for which the fitted second-order rate constant was $0.002 \text{ min}^{-1} \cdot \mu\text{M}^{-1}$, consis-

tent with previous analyses (Fig. 4). The rate constant for digestion in the presence of HABA accords well with the fitted rate constant of $0.032 \text{ min}^{-1} \cdot \mu\text{M}^{-1}$ obtained from analysis of the kinetics of spectral enhancement. In marked contrast to biotin therefore, HABA, which occupies the same site, enhances the proteolytic susceptibility of this region of the protein. The fragmentation pattern is the same as that seen for proteolysis in the absence of ligands, and there is no reason to believe that proteolysis is occurring at different bonds. Thus, a ligand that occupies the same site as biotin has a dramatically different effect—HABA enhances susceptibility of the 3–4 loop by an order of magnitude, whereas biotin protects it completely.

Discussion

The limited proteolytic attack of avidin by proteinase K, the total prevention of this event by biotin, and the enhancement of the process by HABA can be interpreted in terms of the structure of apo- and holoavidin (Livnah et al., 1993a; Pugliese et al., 1994). The factors that influence susceptibility of native proteins to proteolytic attack are largely unknown. A priori, one might expect a combination of accessibility and flexibility to be primary influences. Accordingly, the structures of apo- and holoavidin were analyzed by the algorithms developed by Hubbard et al. (1992). The analysis for apoavidin is shown in Figure 6. The predictions for holoavidin are not substantially different, as would be expected because the two structures (Brookhaven codes 1AVD and 1AVE) possess similar conformations with an overall RMS deviation (RMSD) of 0.53 \AA between the two functional tetrameric units. A superposition of two monomers from the apo and holo forms are shown in Figure 7A and Kinemage 2, with the 3–4 loop highlighted. This loop displays a high accessibility to solvent, protrudes from the bulk of the avidin molecule (even in the tetramer), is situated within one of the most flexible regions of the molecule, but possesses no secondary structure. Thus, this region of the protein is characterized by a strong prediction of proteolytic susceptibility.

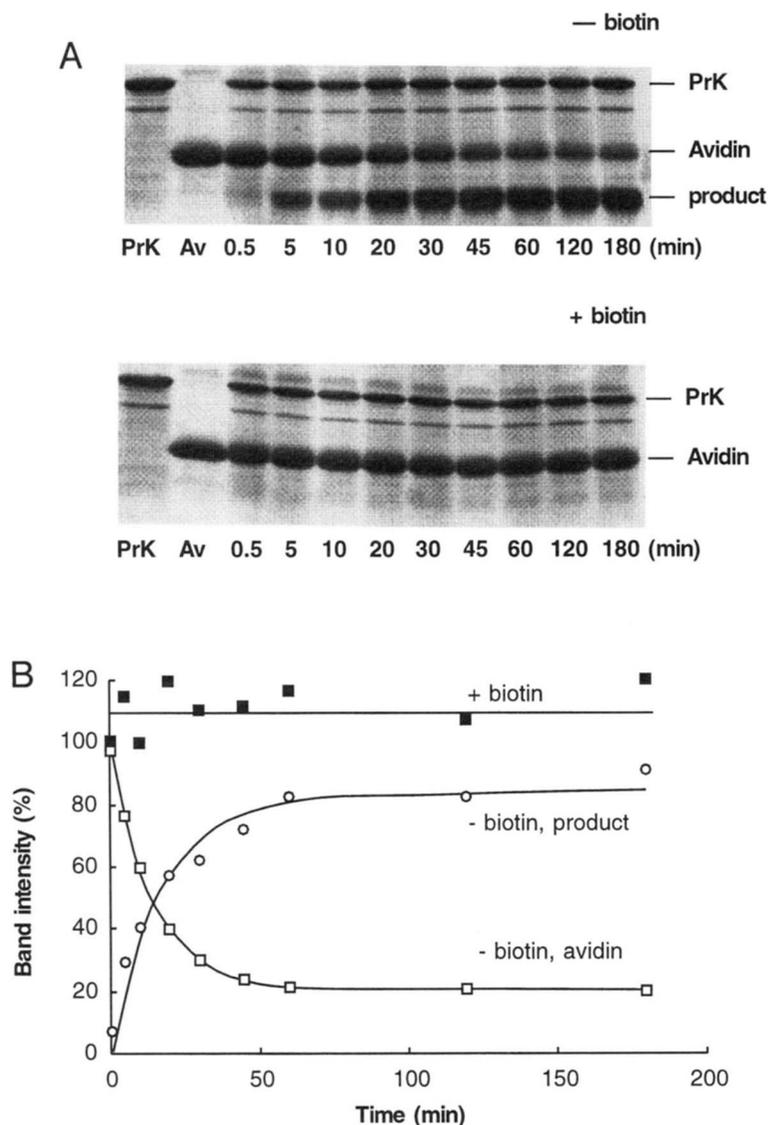


Fig. 4. Effect of biotin on avidin proteolysis. Avidin ($120 \mu\text{M}$ monomer) was digested with proteinase K ($30 \mu\text{M}$) for different times, after which the reaction was stopped by precipitation with 10% (w/v) trichloroacetic acid. Precipitated proteins were resolved on reducing SDS-PAGE, stained with Coomassie blue, and scanned by laser densitometry (**A**). Band intensities were normalized to a constant loading and fitted as a single-exponential decay. Data are presented (**B**) as the experimental data and theoretical fitted curve for disappearance of apoavidin (\square) and appearance of apoavidin digestion product (\circ); in a separate digestion, biotin was included at a final concentration of $600 \mu\text{M}$ (\blacksquare).

Given the similar nick-site predictions for apo- and holoavidin, the dramatic difference in proteolytic susceptibility between the two forms is surprising. The obvious source of explanation of this phenomenon is the ligand. Biotin binding to apoavidin protects against the action of denaturing agents, and the T_m is increased from 85°C to 132°C when biotin is bound (Donovan & Ross, 1973; Green, 1975). Clearly, the binding of the natural ligand induces considerable additional stability in the protein. Biotin binding is accompanied by a rigidification of the binding site and a marked change in the environment and conformational freedom of the tryptophan residues that line the biotin-binding site (Kinemage 2), notably Trp 70, but including Trp 97 and a tryptophan residue from a neighboring subunit. Analysis of the dynamic polarization of tryptophan fluorescence also provides evidence for loss of segmental mobility when biotin is bound (Mei et al., 1994). Biotin protects tryptophan residues from quenching agents, and modification of tryptophan residues, notably Trp 70, destroys the capacity of the avidin molecule to bind biotin (Kurzban et al., 1989).

The similarity in structure between the crystal forms of apo- and holoavidin may therefore reflect a constraint in the crystal structure that is not present in solution and that conceals the ability of the 3–4 loop to adopt other conformations. Evidence for this is derived from the crystal structure of apoavidin, where the 3–4 loop is stabilized by interaction between Ser 41 and Lys 111 in a symmetry-related molecule. Furthermore, the aperture to the biotin-binding site, which is flanked by the 3–4 loop, is not large enough to accommodate the biotin molecule. This lends extra credence to this region of the molecule being flexible and thus susceptible to proteolysis. Indeed, in a different crystal form (1AVI; Livnah et al., 1993a), the 3–4 loop is not visible, suggesting substantial mobility in this region of the molecule. Additionally, in the structurally homologous streptavidin, the 3–4 loop cannot be visualized, presumably because of disorder and loop flexibility (Weber et al., 1989). One outcome of this analysis is therefore a caution against the role of symmetry-related contacts in rigidification of otherwise disordered and therefore putative proteolytic target segments. More impor-

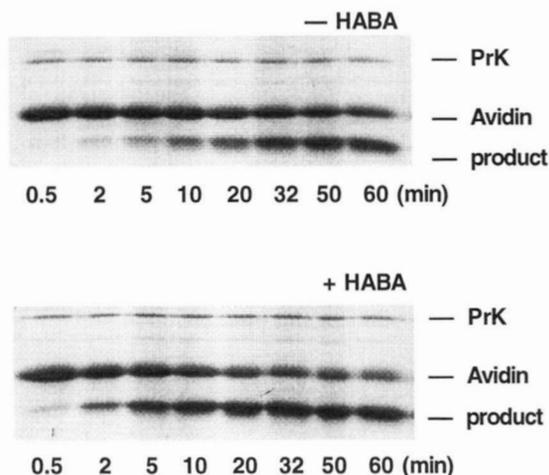


Fig. 5. Effect of HABA on avidin proteolysis. Avidin ($120 \mu\text{M}$ monomer) was digested with proteinase K ($5 \mu\text{M}$) in the absence or presence of $100 \mu\text{M}$ HABA for different times, after which the reaction was stopped by precipitation with 10% (w/v) trichloroacetic acid. Precipitated proteins were resolved on reducing SDS-PAGE, stained with Coomassie blue, and scanned by laser densitometry. Band intensity/time data were analyzed by nonlinear curve fitting to derive the rate constants for proteolysis (see text).

tantly, the analysis emphasizes the role of flexibility in creation of nick sites.

Structural analysis of the biotin-bound holoprotein reveals a number of important details, illustrated in Figure 7B and Kine-

mage 2. The 3–4 loop itself makes a number of van der Waals contacts with biotin, including two good hydrogen bonds formed from main-chain amide groups to the valerate side chain of the ligand (Pugliese et al., 1994). Thus, the loop may be considered as a “lid” to the binding site, closing down on top of the bound ligand and making good interactions with it. Consequently, the loop is tightly constrained by biotin, reducing its conformational flexibility and hence its susceptibility to proteolysis by proteinase K, because it will be less able to adopt the prerequisite substrate conformation.

Why should proteolysis of the 3–4 loop result in enhanced binding of HABA to avidin? It seems logical that the covalent disruption of the 3–4 loop would result in a dramatic change in the conformational space available to it, allowing the ligand to bind more efficiently within the biotin-binding site. HABA binding to unproteolyzed avidin is accompanied by a rigidification of two residues of the otherwise disordered 3–4 loop (Livnah et al., 1993b). This conjecture is supported by the observation that the extinction coefficient at 500 nm is enhanced, consistent with HABA being in a more hydrophobic environment (Baxter, 1964). Indeed, the hydroxyphenol ring of HABA comes to lie between the aromatic rings of Phe 72 and Trp 70 in the HABA–avidin complex (Livnah et al., 1993b) and these stacking interactions may be enhanced in the proteolyzed complex. There is some further evidence to support this, which also explains the apparently anomalous enhancement of proteolysis produced by HABA binding. Given that biotin-bound holoavidin is refractory to proteolysis, and that this is ascribed to the interactions that biotin makes with the susceptible loop, one would expect HABA to offer some protection against proteol-

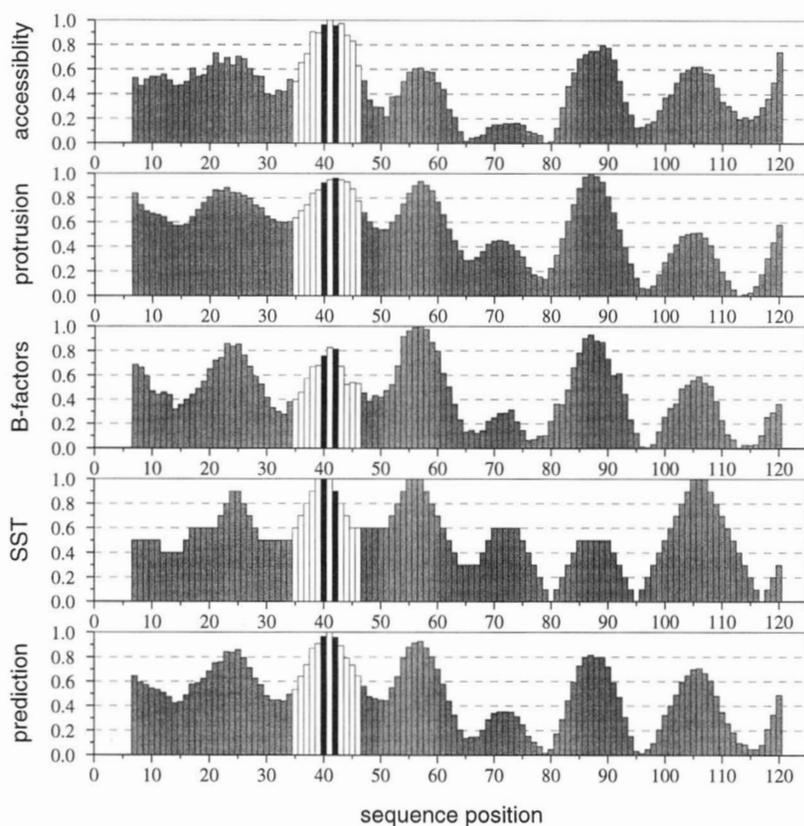


Fig. 6. Nick-site prediction and structural analysis of apoavidin. Three-dimensional structures of the apoavidin tetramer (Brookhaven code 1AVE) were analyzed by the algorithms developed by Hubbard et al. (1992). Data are presented as separate profiles for accessibility, protrusion, *B*-value, and secondary structure parameters, which are then aggregated into a composite score in the bottom panel (see Materials and methods). Data are presented for a single subunit – the analyses for the other three subunits are essentially identical. The 3–4 loop is highlighted in white and the nick sites at Thr 40 and Asn 42 are highlighted in black.

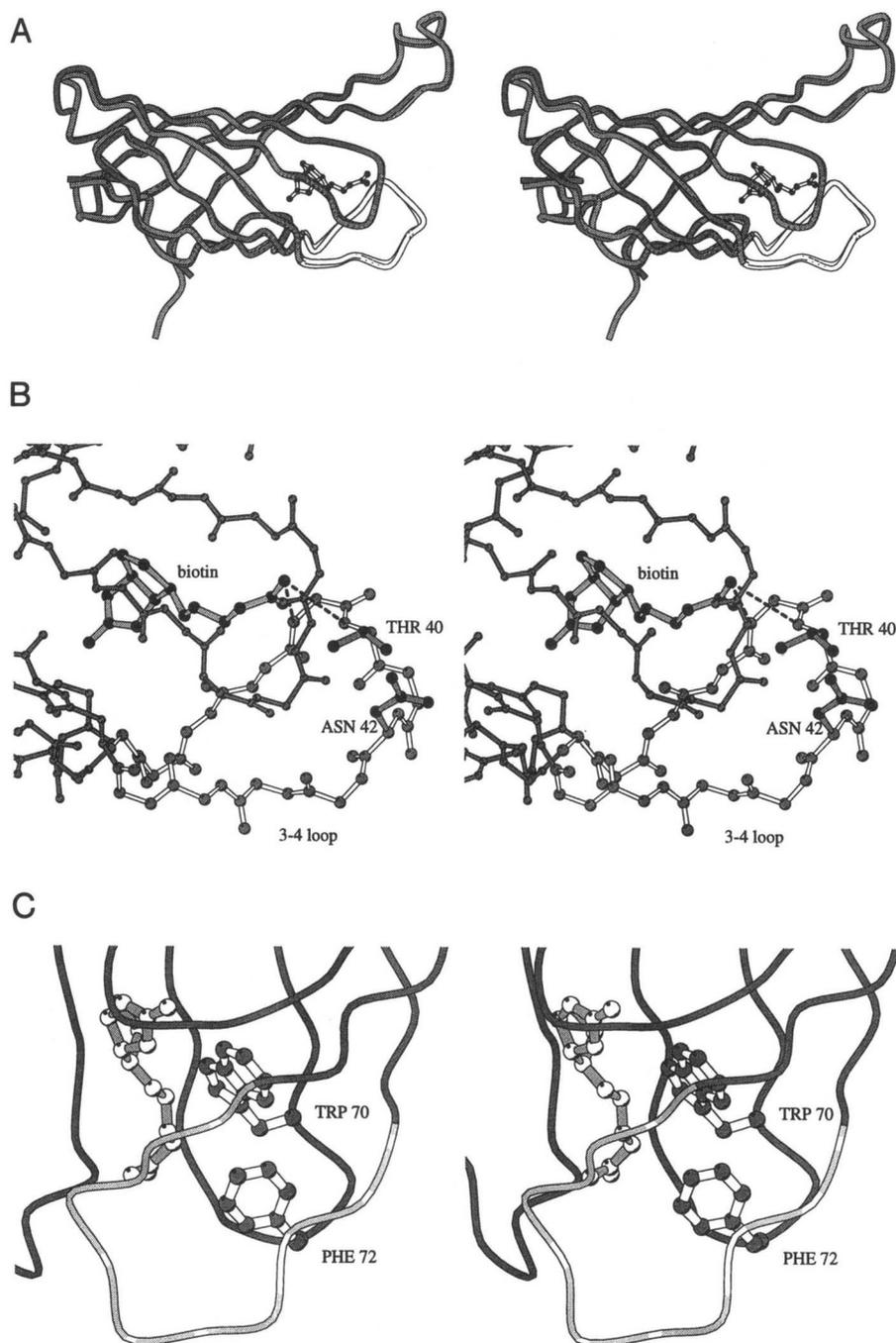


Fig. 7. Superposition of the structures of apo- and holoavidin. **A:** Following a superposition of the main-chain atoms of a single subunit of apo- and holoavidin, a MOLSCRIPT (Kraulis, 1991) trace over the main chain is shown, with the apo-form (1AVE) shown with lighter gray bonds. The loop 3-4, highlighted in white, is the site of proteinase K digestion. **B:** Close-up view of the conformation of the 3-4 loop in the holo protein is shown, with the side chains of Thr 40 and Asn 42 indicated, as is biotin, in the conformation as bound to the holo-protein. Two hydrogen bonds made by one of the biotin valerate oxygens to the amide groups of residues Ala 39 and Thr 40 are indicated by dotted lines. **C:** Spatial relationship between Phe 72 and the 3-4 loop is shown. In the HABA-bound holo form, the hydroxyphenol group of the ligand comes to lie between the aromatic rings of Trp 70 and Phe 72, forcing the Phe to move 0.9 Å toward the loop.

ysis too, and certainly not to enhance it. However, these interactions with the loop could be crucial. The two hydrogen bonds made by the amide groups of Ala 39 and Thr 40 are lost in the HABA complex, and no compensatory additional hydrogen bonds are made. Furthermore, a major change is observed in the position of residue Phe 72 compared to biotin-avidin. As shown in Figure 7C and Kinemage 2, this residue appears crucial to the 3-4 loop conformation, lying directly beneath it and making many contacts to it. This shift in side-chain conformation toward the loop might force the loop out toward the solvent, enhancing the rate of proteolysis by compelling the loop to adopt, or be restricted to, a more accessible range of conformations.

In the apoprotein, the loop is totally unconstrained and will presumably take up many more conformations. HABA binding may reduce these loop excursions and restrict it to more proteolytically susceptible conformations. However, this hypothesis is unproven and requires further investigation of the constraining effects on loops and their dynamics that are imposed by protein structure.

Proteinase K has been used as a conformational probe for avidin in unliganded and complexed states. Although the effects of biotin were unsurprising and consistent with crystallographic data, the data on enhanced HABA binding and on HABA-induced increase in proteolytic susceptibility were unexpected.

It seems likely that HABA is suboptimally bound to the biotin site, and that relaxation of constraints in the 3–4 loop renders the site more accessible. Further, HABA binding precludes some conformations of the 3–4 loop, whereas biotin appears to pin it down almost entirely. Certainly, the 3–4 loop remains flexible in the HABA:avidin complex, and the enhancement in rate of proteolysis may be due to the loop being restricted to a set of conformations that are more compatible with binding to the active site of the proteinase. It may be possible to crystallize apo- and holo-Av^{PrK}, which would permit analysis of the structural consequences of the limited proteolysis. Finally, protein engineering of the 3–4 loop, conceptually feasible because of the exposed and flexible nature of the region, might provide a means to “fine-tune” and modify the ligand specificity and affinity of the ligand-binding site in this molecule. Such modifications may find application in avidin-based technologies (Wilchek & Bayer, 1989; Bayer et al., 1990).

Materials and methods

Chromatographically purified proteinase K was purchased from BDH Ltd. (Poole, Dorset, UK) and HABA was supplied by Sigma Chemical Co. (Poole, Dorset, UK). Biotin was purchased from Pierce and Warriner Ltd. (Chester, Cheshire, UK), and avidin was purified on an iminobiotin affinity matrix also from Pierce and Warriner Ltd. by the method of Heney and Orr (1981). All PAGE was carried out on Protean II mini-gels from Bio-Rad (Hemel Hempstead, UK). General reagents were purchased from BDH Ltd. or Sigma Chemical Co.

Proteolysis of avidin

Stocks of proteinase K and avidin were prepared in 50 mM Tris, 5 mM CaCl₂, pH 8.0, and concentrations were determined using the absorption coefficients $\epsilon_{280} = 40,500$ and 96,000, respectively. Proteolysis was carried out at final concentrations of 3–30 μ M for both protease and substrate in the same buffer at 30 °C. The reaction was stopped by removal of an aliquot to an equal volume of 10% (w/v) trichloroacetic acid, and the resulting precipitate was pelleted at 13,000 RPM in a benchtop microfuge, washed twice with diethylether, and dissolved in sample buffer prior to analysis on a 15% acrylamide SDS-polyacrylamide gel. Assays of proteolysis in the presence of ligands were carried out in a similar manner, except that avidin was preincubated with 0.6 mM biotin or 0.1 mM HABA prior to addition of proteinase K.

Estimation of kinetic constants

Samples from a proteolysis time course were run on SDS-polyacrylamide gels that were then stained in 2% (w/v) Coomassie brilliant blue. The resulting protein bands were quantitated by a Molecular Dynamics Computing Densitometer using ImageQuant software, version 3.0/SCAN version 4.2. To correct for small variations in sample loading, the band intensities were normalized to the proteinase K band. The densitometry data (which we have established to be proportional to amount of protein; results not shown) were analyzed by nonlinear curve fitting of a single exponential function:

$$A_t = A_f + (A_0 - A_f)e^{-kt}, \quad (1)$$

where A_t , the amount at time t , is related to the initial amount (A_0), final amount (A_f), and the first-order rate constant (k). The curve was fitted to the experimental data using the nonlinear optimization functions within Excel 5 (Microsoft).

Peptide sequencing

A 15% acrylamide SDS-polyacrylamide gel of proteinase K-digested avidin was blotted onto PVDF in a Bio-Rad Protean II mini-gel blotting kit at 100 V for 1 h. After staining with Coomassie blue, the bands were cut from the membrane and sequenced on an Applied Biosystems 476 protein sequencer. Yields were back-extrapolated to the first cycle over four or five cycles.

Assay of avidin

Avidin was assayed for its ability to bind the dye HABA (Green, 1965) by measurement of the increase in OD_{500nm} on formation of the avidin-HABA complex. A stock solution of 1 mM HABA was made up in 2 mM NaOH. Aliquots of this (between 2 and 10 μ L) were added to 1 mL of avidin (approx 10 μ M monomer) in 200 mM Tris/HCl, 5 mM CaCl₂, pH 8.0, and the resulting OD_{500nm} recorded. Following the HABA assay the biotin-binding ability of the avidin was measured. Addition of biotin to the avidin-HABA complex results in the stoichiometric displacement of the HABA from the avidin and a corresponding decrease in the OD_{500nm}. Biotin was added in 2- μ L aliquots from a 0.5 mM stock solution in water and the OD_{500nm} again recorded. Avidin digests were prepared by incubating equimolar quantities of proteinase K and avidin for 4 h at 30 °C. Proteolysis was checked by SDS-PAGE. The digests were assayed for avidin activity as above, along with appropriate controls. In a separate experiment, HABA was used at a higher concentration, in order to provide accurate data for the extinction coefficients of free and bound ligand.

Because the avidin (P) and dye (L) concentrations were near to the K_d values, binding was analyzed according to the equation:

$$[PL] = \frac{(K_d + P_t + L_t) - \sqrt{(K_d + P_t + L_t)^2 - 4P_tL_t}}{2}, \quad (2)$$

where $[PL]$ is the concentration of avidin-HABA complex, P_t is the total avidin concentration, L_t is the total HABA concentration, and K_d is the dissociation constant for HABA binding.

The data for HABA binding were fitted to the equation above using the nonlinear optimization functions within Excel 5 (Microsoft), taking into account the absorbance of free and bound HABA. The analysis also permitted optimization of the values for the extinction coefficient of free and bound chromogen. In separate experiments, avidin was preincubated in buffer containing HABA, and proteolysis was initiated by addition of proteinase K. The consequent increase in absorbance was monitored at 500 nm. The absorbance/time data were analyzed as a single exponential by nonlinear curve fitting, also according to Equation 1.

Structural analysis of nick sites

Analyses of avidin structural features were made according to the algorithm of Hubbard et al. (1992) using the Brookhaven

data files 1AVE (apo) and 1AVD (holo). The following conformational parameters were calculated for each residue of both apo and holo tetramers: atomic accessible area using the method of Lee and Richards (1977), protrusion index (Taylor et al., 1983), mean residue crystallographic temperature factors (B -values), and the secondary structure assignments from DSSP (Kabsch & Sander, 1983). For the latter, values of 0.5 and 0.0 were assigned to residues with secondary structures of helix and strand, respectively. All other residues were assigned a value of 1.0 for random coil. Parameters were smoothed over a 10-residue window, and normalized to a scale of 0–1. A final prediction score was then assigned to each residue by averaging all four parameters at each residue position and renormalizing to the same scale. No further window averaging was performed on the aggregated parameters.

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