Affinity Cleavage and Targeted Catalysis of Proteins Using the Avidin–Biotin System†

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ABSTRACT: The avidin–biotin system was used in order to target enzymes to their substrates in complex mixtures of proteins in solution. The approach described here thus mimics natural systems in which enzymes usually act in selective fashion, due, perhaps, to proximity effects. For affinity cleavage studies, biotinyl transferrin was used as a model target substrate. Avidin or streptavidin was then employed to bridge between the biotinylated target protein and a biotinyl protease. Bovine serum albumin was included in the reaction mixtures to assess the level of nonspecific cleavage. In the case of an unbiotinylated target protein, avidin could be used to inhibit the hydrolytic action of the biotinyl protease. In some systems, a biotinyl antibody could be used to direct the avidin-bridged biotinyl protease to an unbiotinylated target antigen. The data support the contention that preferential cleavage reflects two separate phenomena: (i) avidin confers a conformational alteration of the biotinylated target protein, and (ii) the biotinyl protease is targeted (via the avidin bridge) to the proximity of the biotinylated target protein, thereby promoting cleavage of the conformationally altered molecule. This is the first report in which a proteolytic enzyme could be selectively targeted to specifically hydrolyze a defined protein substrate in solutions containing a complex mixture of other proteins. The approach appears to be a general phenomenon for "targeted catalysis", appropriate for other applications, particularly for affinity cleavage and targeted catalysis of cell-based macromolecules.

Avidin and streptavidin are two biotin-binding proteins which exhibit remarkable thermodynamic stability (Green, 1975). Both proteins are tetrameric and comprise one biotin-binding site per monomer. Their stability even increases when biotin is bound.

In experiments designed to identify the portions of avidin which are responsible for the binding of biotin, attempts were made to cleave avidin or streptavidin with proteolytic enzymes (Wilchek & Bayer, 1989; Y. Hiller, E. A. Bayer, and M. Wilchek, unpublished results). As a rule, these attempts proved unsuccessful.

We reasoned that, if proteolytic enzymes would be biotinylated and then interacted with avidin or streptavidin, cleavage may occur by virtue of a first-order reaction at the biotin-binding site of these proteins. However, even under such purportedly favorable reaction conditions, again no cleavage occurred. Furthermore, the complex formed between avidin (or streptavidin) and biotinylated proteases was also not capable of hydrolyzing other target proteins. It was thus possible to use avidin as a means by which proteolytic action could be selectively inhibited, similar to the use of antibodies against lysozyme to prevent hydrolysis of polysaccharides.

On the other hand, when avidin was incubated with subsaturating amounts of a biotinylated protease, such that free biotin-binding sites were available for further interaction, the resulting complex was capable of selective hydrolysis of biotinylated target proteins. The observed specificity is achieved by virtue of the avidin bridge formed between (and the resultant proximity of) the two biotinylated species. Thus, a new approach is introduced for site-directed proteolysis, or affinity cleavage, of target proteins which have been prebiotinylated. Supplemental experiments indicated that the approach may also be of general applicability for targeted catalysis using nonproteolytic enzymes.

Experimental Procedures

Materials. Egg-white avidin was a generous gift of Society Cooperative Belovo (Bastogne, Belgium). Streptavidin was purified from culture filtrates of *Streptomyces avidinii* using an improved iminobiotin–Sepharose column as described previously (Bayer et al., 1990). All other proteins and biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Chemical reagents were of the highest analytical grade available.

Slab Gel Electrophoresis. SDS–PAGE was performed on 10% gels as described previously (Bayer et al., 1987). Samples were diluted with 0.5 volume of sample buffer which contained 9% (w/v) sodium dodecyl sulfate (SDS),[1] 30% (v/v) glycerol, and 0.2% (w/v) bromophenol blue in 62.5 mM Tris-HCl buffer (pH 6.8). 2-Mercaptoethanol was not added to samples. The samples were boiled for 10 min prior to electrophoresis.

Biotinylation Procedures. The proteins used in this study were biotinylated by conventional biotinylating methods using BNHS[1] as described previously (Bayer & Wilchek, 1990a). With the exception of pepsin, the proteases (35 mg of each) were each dissolved in 0.1 M sodium bicarbonate buffer, pH 7.5 (10 mL). An equivalent amount of pepsin was dissolved in 10 mL of 0.1 M sodium phosphate buffer, pH 5.5. A solution of BNHS (3.4 mg dissolved in 1 mL of dimethylformamide) was then introduced to the respective protease solution. Following a 3-h incubation period at room temperature, the proteins were dialyzed overnight at 4 °C against PBS (pepsin was dialyzed and maintained in phosphate buffer, pH 5.5) with three buffer changes. The biotinyl proteases were stored in aliquots at −20 °C.

Human transferrin was biotinylated in a similar manner. A 3.8-mg sample of the protein was dissolved in 0.9 mL of

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[1] Abbreviations: ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); BNHS, biotinyl *N*-hydroxysuccinimide ester; BSA, bovine serum albumin; PBS, phosphate-buffered saline, pH 7.4; SDS–PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

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0.1 M sodium bicarbonate buffer, pH 7.5. BNHS solution (170 μL, 1.7 mg/mL in dimethylformamide) was added to yield a 10-fold molar excess of reagent per protein, and the reaction was carried out for 1 h at room temperature. Dialysis was performed against PBS as described above. Biotinyl transferrin was stored at −20 °C in aliquots at a final concentration of 0.9 mg/mL. Asialofetuin was biotinylated in an identical manner using the same molar excess of BNHS.

Galactose oxidase (0.25 mg) was dissolved in 1 mL of the same buffer and reacted with a 30-fold molar excess of BNHS (1.3 μL, 34 mg/mL in dimethylformamide). The enzyme was treated further as described above for the other biotinylated proteins.

The precise details for biotinylating anti-transferrin antibodies have been published previously (Bayer & Wilchek, 1990b).

The extent of biotinylation of a given preparation was determined qualitatively by dot blot enzyme assay and quantitatively by affinity chromatography on avidin–Sepharose columns (Bayer & Wilchek, 1990a).

Avidin-Induced Termination of Biotinyl Protease Action. To a mixed protein sample containing 2 mg/mL BSA and 0.2 mg/mL unbiotinylated transferrin was added 50 μg/mL biotinyl chymotrypsin (final concentrations in each case). After a desired period of time (see Results), the reaction mixture was brought to a final concentration of 0.3 mg/mL avidin. The reaction was allowed to continue at 23 °C for varying time intervals, and the extent of degradation of the target proteins was determined by SDS–PAGE. Control samples consisted of unbiotinylated chymotrypsin or biotin-blocked avidin (10-fold molar excess of biotin or biocytin).

Preparation of Preformed Avidin–Biotinyl Galactose Oxidase Complexes. Preformed complexes containing biotinyl galactose oxidase and avidin were prepared by incubating the desired molar ratios (as indicated later in the text) of the proteins in PBS. The interaction was carried out for 4 h at 23 °C prior to introduction into the experimental system.

Enzyme Assays. Pepsin activity was determined by the hemoglobin degradation assay as described in the 1977 Worthington Enzyme Manual (Worthington Biochemical Corp., Freehold, NJ). Chymotrypsin and proteinase K were assayed by potentiometric titration using N^{α}-acetyl-L-tyrosine ethyl ester as substrate.

The enzymatic activity of galactose oxidase and its complexes with avidin was determined spectrophotometrically in 96-well microtiter plates. A 90-μL aliquot of substrate solution, comprising 0.2 mg/mL asialofetuin (either native or biotinylated), 0.1 mg/mL ABTS, and 4 units/mL peroxidase in PBS, was added to each well which included 10 μL of the galactose oxidase containing sample. The extent of oxidation was monitored at fixed time intervals at 405 nm in an ELISA reader (Model EL-310; Biotek Instrument, Inc., Burlington, VT).

Protocol for Targeted Catalysis. Targeted catalysis was tested by applying the desired concentrations (as indicated later in the text) of preformed complexes (consisting of avidin premixed with biotinyl galactose oxidase) to reaction solutions containing biotinyl asialofetuin as substrate. Control experiments included asialofetuin instead of the biotinylated substrate and/or biotin-blocked preformed complexes.

Proteolytic Reaction Conditions. In order to test various concentrations of the reactants, stock solutions were introduced sequentially to 1.5-mL Eppendorf reaction vials. The protocol usually included an unbiotinylated protein (BSA), a biotinylated target protein, avidin, and a biotinylated protease. BSA was added to the samples as a combined indicator for, and macromolecular buffer of, nonspecific (untargeted) cleavage.

A typical example of an affinity cleavage experiment consisted of the following reactants, added in succession to yield the designated final concentrations in a final volume of 50 μL: 2 mg/mL BSA (30 nmol/mL), 0.2 mg/mL biotinyl transferrin (2.5 nmol/mL), 0.316 mg/mL avidin (4.7 nmol/mL), and 5 μg/mL biotinyl chymotrypsin (0.2 nmol/mL). After a 5-h incubation period, sample buffer (25 μL) was added to the reaction vial, and 30 μL was subjected to SDS–PAGE.

In any given experiment, the duration of the reaction and the relative concentrations of avidin and/or the desired biotinyl protease were varied in an attempt to achieve the optimal effect regarding selective proteolytic degradation of the target protein. In parallel, control samples were included which comprised the substitution of one or more of the designated reactants with an equivalent amount of its unbiotinylated counterpart and/or biotin-blocked avidin (containing a 10-fold excess of either biotin or biocytin). In some cases, an *unbiotinylated* target protein (transferrin as the model in these experiments) was interacted with a *biotinylated* antibody (polyclonal anti-transferrin which served as the targeting device) followed by successive incubations with avidin and the biotinyl protease.

Another strategy was also examined in some experiments wherein preformed complexes of avidin and the biotinyl protease were prepared by their combined incubation (30 min at room temperature) prior to application to the reaction mixture containing the biotinylated target protein (or the unbiotinylated target protein combined with the biotinylated antibody). In an extension of this approach, we also examined the preparation of preformed trifunctional heterocomplexes, which consisted of a mixture of avidin with a biotinylated antibody *and* a biotinyl protease (performed at 4 °C so as to prevent proteolytic digestion of the antibody in the complex); this mixture was then introduced at 23 °C to the solution which contained the unbiotinylated target antigen. Various concentrations and ratios of these reactants were examined.

Results

Biotinylation of Proteolytic Enzymes. For most of the proteases examined, the standard biotinylation procedure resulted in excellent levels of biotinylation as determined by dot blots. In the case of the three proteases which were investigated in depth in the present work, the interaction of their biotinyl derivatives with avidin was quantified by using an avidin-containing affinity resin. Thus, for biotinyl pepsin, biotinyl proteinase K, and biotinyl chymotrypsin, more than 95% of the respective biotinylated preparation was selectively retained on the avidin column, as determined by the amount of enzyme activity applied to the avidin column versus the amount which appeared in effluent fractions.

The specific activity of the free biotinylated proteases ranged between 60% and 80% that of the corresponding unbiotinylated protein. Under the conditions described in this paper, none of the biotinyl proteases succeeded in degrading avidin or streptavidin. It is interesting to note that successful biotinylation as described here infers that the biotin moiety was covalently linked in a stable form and was not subject to removal by proteolytic action of the protease itself (under the reaction conditions, storage conditions, and time intervals mentioned above).

Termination of Proteolytic Activity by Avidin. When biotin was covalently attached to a protease, the resulting derivative was capable of degrading proteins in a manner similar to that of the unbiotinylated protease. The proteolytic action of the

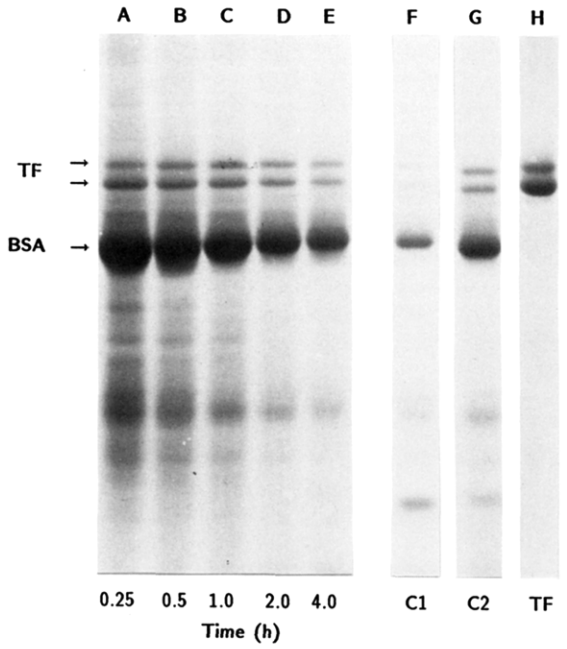

FIGURE 1: Effect of avidin on proteolytic action of biotinyl chymotrypsin. Avidin (15 μg) was added at the indicated time intervals to a reaction mixture (50 μL) which contained BSA and transferrin (100 and 10 μg, respectively) as target proteins and biotinyl chymotrypsin (25 ng) as the biotinyl protease. After 4 h, the samples were boiled in the presence of SDS and subjected to SDS–PAGE. Thus, each sample was incubated with biotinyl chymotrypsin for the same period of time (4 h), but the length of time that the given sample was incubated in the presence of avidin (i.e., 4 h *minus* the indicated time) was different. For example, the sample in lane C was incubated for 1 h with the biotinyl protease without avidin and then another 3 h after avidin was added. The control (C1) in lane F contained unbiotinylated chymotrypsin instead of the biotinyl protease in the reaction mixture (avidin was added after 15 min), and for the control (C2) in lane G, biotin-blocked avidin (instead of the native egg white protein) was added to the reaction mixture after 15 min of proteolytic action by biotinyl chymotrypsin. Lane H gives a transferrin standard as a marker.

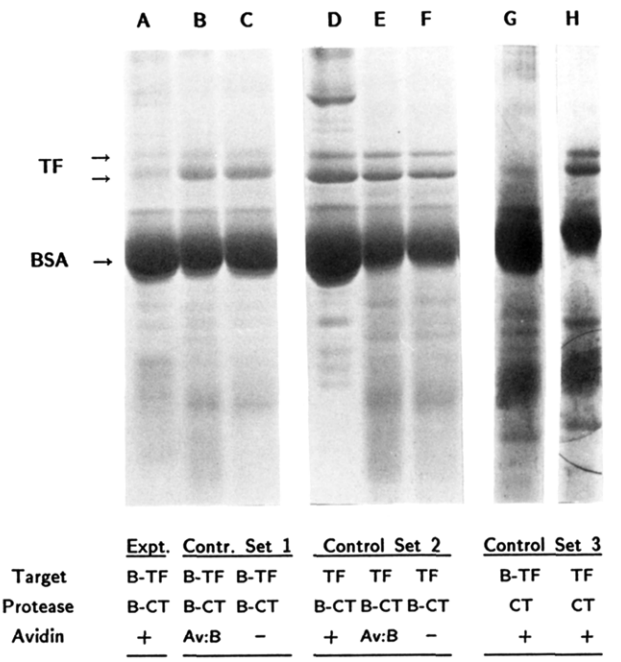

FIGURE 2: Affinity cleavage of biotinyl transferrin by avidin and biotinyl chymotrypsin. To a solution containing biotinyl transferrin as a target protein and BSA as a combined indicator and buffer protein were added in succession avidin (or biotin-blocked avidin) and biotinyl chymotrypsin. After 5 h, the samples were boiled in the presence of SDS and subjected to SDS–PAGE. The final concentrations of the reactants in the experiment shown in lane A were 0.2 mg/mL biotinyl transferrin, 2 mg/ml BSA, 0.316 mg/mL avidin, and 5 μg/mL biotinyl chymotrypsin. The final volume of the reaction mixture was 50 μL. Lanes A–C and G contained biotinyl transferrin (B-TF) as the target protein; lanes D–F and H contained unbiotinylated transferrin (TF) instead. In lanes B and E, biotin-blocked avidin (Av:B) was substituted for the biotin-free protein; avidin was not included in lanes C and F. Lanes G and H contained unbiotinylated chymotrypsin (CT) instead of the biotinylated protease (B-CT).

biotinyl proteases could be arrested by introducing avidin into the solution. Biotinyl chymotrypsin was included in a solution which contained a mixture of BSA and transferrin, and proteolysis was allowed to proceed at 37 °C. At the indicated time intervals (Figure 1), avidin was added to the samples. After a 4-h reaction period, all of the samples were processed for SDS–PAGE (boiled for 5 min in SDS-containing sample buffer). One control consisted of the same reaction mixture with unbiotinylated chymotrypsin substituted for the biotinyl protease; in another, biotin-blocked avidin was substituted for the native biotin-binding protein.

As can be seen in Figure 1, if avidin was added after relatively short incubation periods to the solution which contained the biotinyl protease, much greater amounts of the target proteins (BSA and transferrin) remained intact (i.e., less was degraded by the protease). At longer incubation periods in the absence of avidin, the degradation of the target proteins continued until avidin was added. This clearly indicates that the presence of avidin efficiently inhibits the proteolytic action of the biotinyl protease on proteinaceous substrates. The unbiotinylated protease was unaffected by the presence of avidin (lane F). Likewise, biotin-blocked avidin failed to affect the observed proteolytic action of the biotinylated protease (lane G, compare with lane A). Interestingly, the avidin-complexed protease was still active in hydrolyzing small model substrates (e.g., N^{α}-acetyl-L-tyrosine ethyl ester).

Targeted Proteolysis of Model Proteins in Solution. Incubation of biotinyl transferrin as a model target protein in the presence of avidin and biotinyl chymotrypsin led to an enhancement of its proteolytic degradation compared to an unbiotinylated protein (BSA) included as a macromolecular buffer and indicator of nonspecific cleavage (Figure 2, lane A). Virtually identical results were achieved using bacterial streptavidin instead of egg white avidin (data not shown).

Three sets of control experiments were performed in order to determine the factors which contribute to the observed enhancement. In the first set, the role of avidin was examined either by blocking its biotin-binding site (lane B) or by its exclusion from the reaction mixture (lane C). In both cases, markedly less biotinyl substrate was proteolysed, thus demonstrating that free avidin is essential for affinity cleavage. It is also evident that in the absence of avidin (or in the presence of biobin-blocked avidin) the unbiotinylated buffer protein (BSA) has been extensively degraded, compared with the interaction observed in lane A.

In the second control set, the requirement for biotinylation of the target substrate was evaluated. In this context, unbiotinylated transferrin failed to undergo preferential proteolysis (see lanes D–F) in the presence of avidin. This result emphasizes the importance of avidin–biotin complex formation for selective cleavage of the biotinyl substrate observed in lane A.

Control set 3 was designed to estimate the requirement for biotinylation of the protease. Surprisingly, use of native (unbiotinylated) chymotrypsin on biotinyl transferrin (complexed with avidin) also resulted in some enhancement of proteolysis (compare lanes G and A). This result again suggests the critical role played by the avidin–biotin complex in

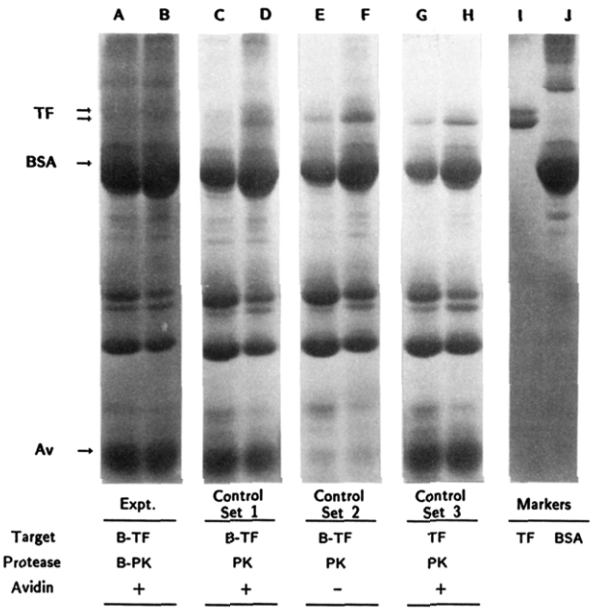

FIGURE 3: Affinity cleavage of biotinyl transferrin by avidin and biotinyl proteinase K. The reaction was performed in a manner similar to that described for biotinyl chymotrypsin in the legend to Figure 2; the final concentration of avidin was 0.5 mg/mL; two different concentrations of biotinyl proteinase K (0.5 and 1.0 μg/mL) were examined. The final volume of the reaction mixture and final concentrations of biotinyl transferrin and BSA were those given in the legend to Figure 2. Lanes A–F contained biotinyl transferrin (B-TF) as the target protein; lanes G and H contained unbiotinylated transferrin (TF). Lanes A and B contained biotinyl proteinase K (B-PK) at concentrations of 1 and 0.5 μg/mL, respectively; lanes C–H contained unbiotinylated proteinase K (1 μg/mL in lanes C, E, and G; 0.5 μg/mL in lanes D, F, and H). Avidin was not included in lanes E and F. Lanes I and J contained 0.2 mg/mL transferrin and 2 mg/mL BSA, respectively, as markers.

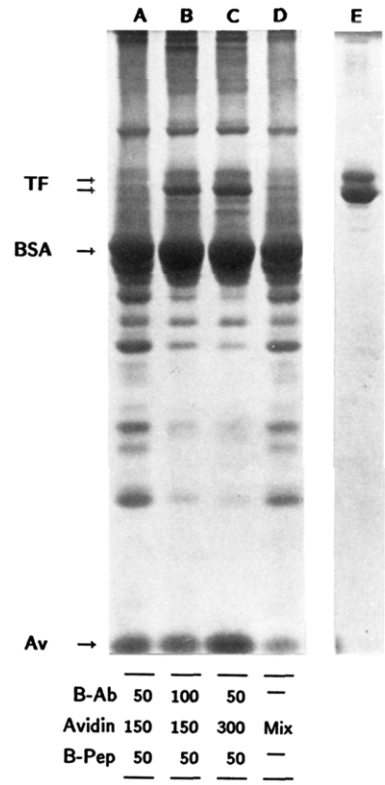

FIGURE 4: Affinity cleavage of an unbiotinylated target protein by avidin and a biotinyl protease, mediated by specific biotinylated antibodies. In lanes A–C, the reaction was performed by sequential addition of the reactants to a mixed solution containing unbiotinylated transferrin (0.2 mg/mL) as the target protein and BSA (2 mg/mL) as an indicator/buffer protein. Stock solutions of biotinyl anti-transferrin antibodies (B-Ab), avidin, and biotinyl pepsin (B-Pep) were then added. The final concentration of biotinyl anti-transferrin antibodies was 0.05 mg/mL in lanes A and C and 0.1 mg/mL in lane B; the final concentration of avidin was 0.15 mg/mL in lanes A and B and 0.3 mg/mL in lane C; that of biotinyl pepsin was 50 ng/mL in all samples. In lane D, to the same target protein mixture were applied preformed complexes (Mix) consisting of biotinyl anti-transferrin antibodies, avidin, and biotinyl pepsin (final concentrations as in lane A). In all cases, the reaction was carried out at 23 °C, pH 5.5; the final volume of the reaction mixture was 50 μL. Proteolysis was terminated after 4 h of incubation by the addition of NaOH. Lane E contained 0.2 mg/mL transferrin as a marker.

the proteolytic reaction; avidin presumably induces a conformational change in the structure of the biotinyl substrate, resulting in its enhanced accessibility to and degradation by the unbiotinylated enzyme (see Discussion). As would be anticipated, if the target protein is not biotinylated, native chymotrypsin exhibits no preference for its degradation (lane H). The major observable difference between the action of chymotrypsin and that of biotinyl chymotrypsin toward this particular sample is that relatively more of the unbiotinylated buffer protein (BSA) appears to be degraded (compare lanes D and H). This result underscores the phenomenon described in Figure 1, that avidin tends to inhibit the proteolysis of unbiotinylated target proteins, but as shown here, it allows the preferential degradation of a biotinylated target protein.

Similar results were obtained using biotinyl proteinase K instead of biotinyl chymotrypsin in an identical approach (Figure 3). In this case, the system exhibited selective proteolysis of biotinyl transferrin by biotinyl proteinase K together with avidin; although the unbiotinylated protease also preferentially degraded the biotinylated target protein, biotinyl proteinase K was more efficient (compare lanes A and B with lanes C and D; note also the preferential degradation of biotinyl transferrin versus that of BSA in the former lanes). Control samples (which included unbiotinylated target proteins, the absence of avidin, etc.) failed to exhibit such selective degradation.

Similar results for both of these proteolytic enzymes were obtained for other target proteins, e.g., for biotinylated antibodies (data not shown). The results therefore appear to be of a general nature for other proteins. Using biotinyl chymotrypsin and biotinyl proteinase K, however, we were unable to demonstrate the applicability of affinity cleavage for an unbiotinylated target protein by means of a biotinylated binder (i.e., antibody). Nonetheless, preferential degradation of an unbiotinylated target protein could be demonstrated by using a biotinylated preparation of relevant antibodies and biotinyl pepsin as the biotinylated protease. For this purpose, unbiotinylated transferrin was subjected to sequential interaction with biotinyl anti-transferrin antibodies, avidin, and biotinyl pepsin (Figure 4). The precise ratio of each of the reactants was of primary importance to the observed hydrolysis of the target protein. If the amount of biotinylated antibody or avidin was too high or too low (e.g., see lanes B and C), little hydrolysis of transferrin could be detected. Using the optimal concentrations of biotinyl antibody, avidin, and biotinyl pepsin, preformed complexes of the three reactants (prepared by combining at 4 °C the appropriate stock solutions 1 h prior to introduction to the target protein mixture and incubation at 23 °C) could be used to effect a remarkably similar preferential degradation of transferrin (lane D). Highly reduced levels of selective cleavage of transferrin were observed for controls, e.g., using biotin-blocked avidin (data not shown).

Inhibition of Biotinyl Galactose Oxidase by Avidin. In order to examine whether the results observed for biotinyl proteases would be of a more universal nature, we designed

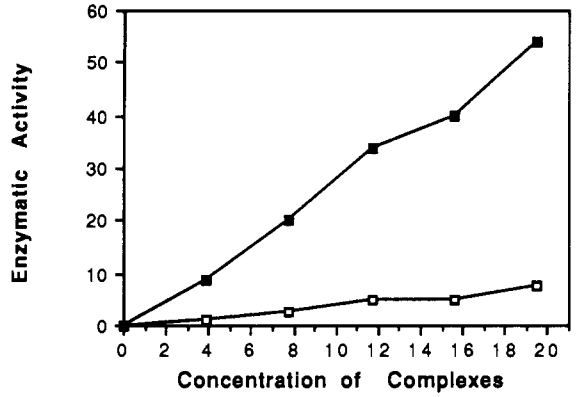

FIGURE 5: Targeted catalysis of biotinyl asialofetuin by avidin–biotinyl galactose oxidase complexes. Complexes containing avidin and biotinyl galactose oxidase (at an initial molar ratio of 10:1) were prepared and applied to a substrate solution containing either asialofetuin (□) or biotinyl asialofetuin (■). Enzyme activity is proportional to the rate of chromophore production assayed at 405 nm; rates are expressed as $\Delta A_{405} \times 10^5 \cdot min^{-1}$. Concentration of complexes is expressed as micrograms of protein per milliliter of reaction mixture.

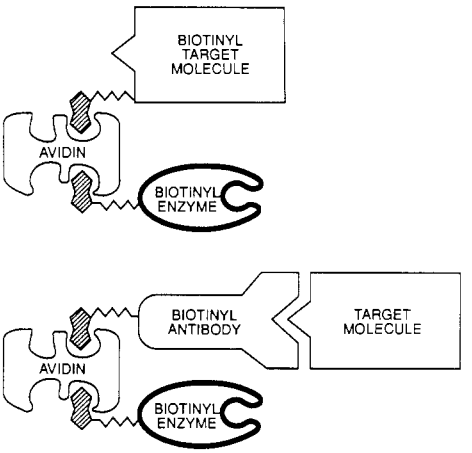

FIGURE 6: Schematic description of the concept of targeted catalysis. In the upper diagram, avidin is used to bridge between a biotinyl target molecule and a biotinyl enzyme. In the lower diagram, avidin serves to cross-link a biotinylated enzyme and a biotinylated binder (e.g., antibody, lectin, or hormone), and the resultant heterocomplex would recognize specifically and selectively catalyze a native target molecule (e.g., antigen, glycoconjugate, or effector, respectively).

similar experiments using nonproteolytic enzymes. For these studies, biotinyl galactose oxidase was chosen as a model enzyme, and asialofetuin was selected as a model glycoprotein substrate.

Biotinylation of galactose oxidase was accomplished by using a large excess of BNHS in order to achieve optimal inhibition of the biotinylated enzyme upon complexation with avidin. Both heavy biotinylation of the enzyme (under the conditions described here, the biotinylated galactose oxidase retains full enzymatic activity) and extensive complexation with avidin were required for effective inhibition of oxidation of the native (unbiotinylated) target glycoprotein (asialofetuin).

Preformed complexes prepared with a 2-fold molar excess of avidin failed to exhibit a strong inhibitory effect on unbiotinylated asialofetuin. In contrast, complexes prepared using a large excess (e.g., 10-fold) of avidin over biotinyl galactose oxidase underwent effective inhibition of enzyme activity: over 90% reduction of the initial activity (exhibited by the uncomplexed enzyme) was observed with asialofetuin as a substrate (data not shown). Similar to the results described above for the biotinyl proteases, the enzymatic activity of the avidin-complexed biotinyl galactose oxidase was essentially unaffected by the complexation process when a small substrate (e.g., galactose) was used. These observations suggest that the catalytic action of the latter complexes on large substrates is prevented by the inaccessibility of the target to the active site of the enzyme.

Targeted Catalysis Using Preformed Complexes. Preformed complexes containing a 10-fold molar excess of avidin over biotinyl galactose oxidase were used to further study the specificity of the complexed enzyme toward the native or biotinylated model substrate (asialofetuin). As can be seen in Figure 5, substantially increased oxidation rates (up to 8-fold higher) were obtained when biotinyl asialofetuin was used compared to the native (unbiotinylated) substrate. If the same complexes were blocked with biotin, such preferential oxidation was not observed, demonstrating that the selectivity is dependent on the recognition properties between the avidin in the complexes and the biotin moiety of the substrate. The levels of oxidation rates exhibited by the free (uncomplexed) biotinyl galactose oxidase were very similar for both the biotinylated and unbiotinylated substrates (not shown), again supporting the role of avidin in the targeting. Preliminary observations indicate that other biotinyl enzymes (e.g., lipase, lactoperoxidase, and transglutaminase) are also affected in a similar manner by avidin and a biotinyl target protein, suggesting that targeted catalysis can be developed into a general approach.

Discussion

In the last decade, the avidin–biotin system has proved of enormous utility and versatility for a variety of different applications (Bayer & Wilchek, 1978, 1980; Wilchek & Bayer, 1984). The scope of application continues to broaden throughout the years, and the description of novel applications continues to appear in the literature (Wilchek & Bayer, 1989, 1990).

The rationale behind the present work is based on the original observation that, although avidin inhibits the action of biotinylated proteases on native proteins, it promotes their selective action on biotinylated target proteins. By virtue of this single observation, we were able to develop procedures through which (i) proteolytic action can be selectively arrested, (ii) a biotinylated target protein can be preferentially cleaved, and (iii) an unbiotinylated target protein can also be specifically cleaved via interaction with a biotinylated antibody. The phenomena described here appear to be of general application to other (i.e., nonproteolytic) enzymes, suggesting "targeted catalysis" as a general approach.

An approach also termed "affinity cleavage" was recently described in which a chemical cleavage system, consisting of a biotin-derivatized chelator, caused the successful severing of streptavidin into two parts (Hoyer et al., 1990). Unlike the approach presented in the present paper, the latter technique is limited to biotin-binding proteins; if a more general approach is desired, individual substrate–chelator derivatives would have to be synthesized for every class of protein (Schepartz & Cuenoud, 1990).

The general approach for targeted catalysis or affinity cleavage, as described in this paper, is shown schematically in Figure 6. In one case, a biotinyl target molecule is directly recognized by avidin which is bound to or associated with a biotinyl enzyme. In the more general case, a biotinylated antibody (or other binder, e.g., hormone, receptor, lectin) can be used to bind to a target antigen; avidin (or streptavidin) and the biotinyl enzyme are then complexed to the site of the target molecule. In this work, we have demonstrated the feasibility of such an approach using model components; we

employed either sequential application of the affinity system and in some cases mixed preformed heterocomplexes of the relevant reactants. Of course, as outlined earlier [see Table I in Wilchek and Bayer (1988)], other strategies (e.g., the use of avidin–enzyme conjugates) may be used and, in the future, may prove superior to those described here.

Successful implementation of targeted catalysis in general, or affinity cleavage in particular, is highly dependent on the composition of the reaction mixture and a precise set of conditions. Changes in the concentration of a given reactant may alter greatly the extent of selective catalysis or degradation of the target protein. Likewise, the sequence of introduction of the various reactants and the manner in which the complexes are formed are both critical to the efficiency and the specificity of the reactions. The extent of biotinylation of a given component (target, binder, enzyme), the type, stability, and/or source of the enzyme, and the time and conditions of interaction all have their individual effect on the specificity of targeting.

In the case of affinity cleavage, for high levels of specificity, it was found that either the concentration of protease or the conditions for its reaction should be designed for suboptimal operation. Thus, very low levels of proteolytic enzyme (low concentration and long reaction times) and/or poor conditions for proteolysis (e.g., pH 5.5 for pepsin) should be chosen. Otherwise, the intrinsic efficiency of the protease can overcome the constraints imposed by the avidin–biotin system. Likewise, in the case of biotinyl galactose oxidase, highly complexed enzyme is required both for the inhibition of the nonspecific catalysis (i.e., on an unbiotinylated substrate) and for the enhancement of the targeted reaction.

It is interesting that reasonable levels of selectivity could also be obtained with an unbiotinylated protease. One possible explanation for this is that complexation between avidin and the biotinylated target protein may cause conformational deformation of the latter, which subsequently promotes a higher affinity interaction with the protease. This effect may be an "artificial" equivalent to the naturally occurring ubiquitin-mediated system for protein degradation (Hershko, 1988). It should be noted that the observed enhancement may also be dependent on the particular target protein and/or the type of protease used in the experimental system. Moreover, due to the chemical modification of the target and/or steric considerations, the cleavage pattern may differ from that of the unbiotinylated or avidin-blocked system.

Nevertheless, the concept of targeted catalysis is posed here to emulate natural systems. Proteins and enzymes are not simply floating around freely in their native environment but are components of ordered structures in which each enzyme or protein is intrinsically programmed to selectively perform its required task. This study was designed in order to simulate nature by biochemically coupling two protein species (enzyme and target) by means of an avidin–biotin bridge.

Regarding the affinity cleavage of an unbiotinylated target protein via mediation of a biotinylated antibody, two of the three proteases examined failed to yield the desired result. In this case, the reason may simply be that the optimal conditions have not be determined for biotinyl chymotrypsin and biotinyl proteinase K. Another possibility is that, for the latter two proteases, the biotinyl antibody may be totally degraded and further interaction of the unbiotinylated target antigen with the biotinyl protease is aborted. On the other hand, biotinyl pepsin would be expected to cleave only the Fc portion of the antibody, and the remaining fragment would still bind the antigen (Parham, 1986). It is interesting to note that we were able to successfully demonstrate the action of soluble heterocomplexes containing three different components: two biotinylated proteins (antibody and pepsin) cross-linked by the biotin-binding sites of the avidin tetramer.

We would like to emphasize at this point that the results obtained in this paper were achieved for target proteins in the solution phase. It is expected that a more substantive and applicative system will involve the targeted catalysis or affinity cleavage of cell-based target proteins (e.g., biotinylated antibodies and their surface antigenic receptors). For example, in the future, the approach can be used to selectively remove biotinyl antibody molecules bound to antigens on cell surfaces without affecting cell viability. Likewise, the approach can also be used to selectively degrade extracellular matrix proteins to cause the detachment of desired cell types from adherent surfaces with minimal degradation of cellular components. In addition, other types of enzymes, such as the biotinyl galactose oxidase system, can be employed to selectively catalyze a reaction at or near a specific membrane-based target site, e.g., a particular glycoprotein antigen. Work in these directions is currently in progress in our laboratory.

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