A Rapid and Efficient Method for Identifying Photoaffinity Biotinylated Sites within Proteins

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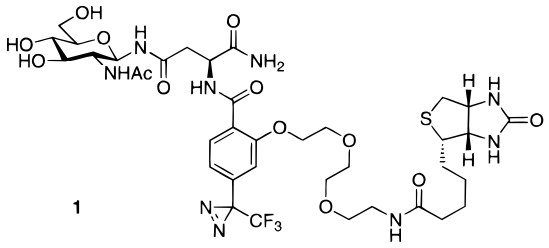
Photoaffinity labeling is a powerful chemical approach in the field of structural biology.[1] The conventional approach, however, usually relies on the synthesis of radiolabeled probes and the identification of radiolabeled fragments in proteins. Here, we report an example of photoaffinity labeling that produces nonradioactive biotinylation of the binding site of the catalytic domain of bovine UDP-galactose:*N*-acetylglucosamine β1,4-galactosyltransferase (GalT, EC 2.4.1.38). The approach yields, for the first time, information on the binding-site residues in this enzyme that have been difficult to obtain using other approaches.

Glycosyltransferases are key enzymes in oligosaccharide biosynthesis, and many recent studies have revealed aspects of their structure and function.[2] Sequence alignments of the cloned glycosyltransferases reveal moderate degrees of sequence homology, and such studies have been useful in determining the conserved topographical aspects in these proteins. However, these comparisons have not localized the substrate binding sites. GalT has been cloned and sequenced.[3] A conventional radioactive diazirine photoprobe has been used for labeling of the acceptor site of GalT but the labeled site was not identified.[4] One possible difficulty may be the relatively large-scale purification of labeled peptides using large quantities of radiolabel.

Our new approach uses a novel biotinyl photoprobe (**1**)[5] to provide a means for radiochemical-free detection of labeled protein on a polyvinylidene difluoride (PVDF) membrane. Small



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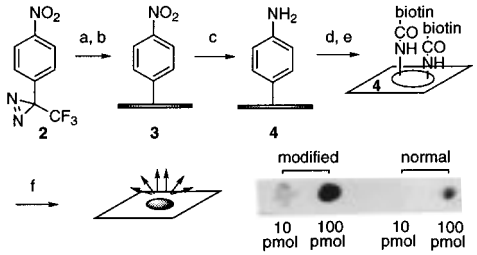
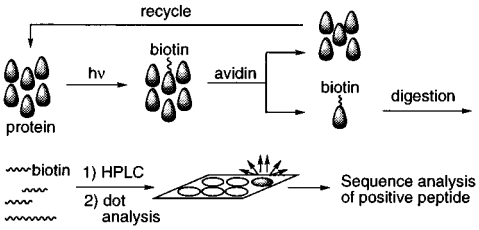
peptides do not bind strongly to the normal PVDF membrane, however, precluding further analysis of the biotin content of proteolytic fragments. Derivatization of the membrane was therefore found to be necessary, and we have developed a surface-modified membrane (**4**) for the immobilization of peptide fragments on the PVDF surface (Scheme 1).

Scheme 1. Aminophenyl PVDF Membrane **4** for the Immobilization of Peptides[a]



[a] Conditions: (a) PVDF membrane, immerse in 0.1 M methanolic solution of **2**, dry; (b) irradiate, 30 W black-light lamp, 30 min; (c) 0.1 M $Na_2S_2O_4$/0.5 M $NaHCO_3$, 0.5 h; (d) dot blot biotin (1 μL, 10 or 100 μM in H_2O) on **4**, dry; (e) overlay EDC−HCl (20 mM in H_2O), overnight; (f) chemiluminescent detection.[5]

Scheme 2. A Strategy for Rapid Identification of GalT Acceptor Site



A commercial PVDF membrane was photochemically modified with a nitrophenyldiazirine (**2**)[6] followed by reduction of the nitro group to give the aminophenyl membrane **4**. For evaluation of the peptide retention properties of **4**, the carboxylic acid group of biotin was coupled to the membrane amino group using a carbodiimide method. The extent of immobilization was assessed by chemiluminescence. The intensity of chemiluminescence due to biotin associated with the membrane was found to be much greater than for the unmodified PVDF membrane; 10 pmol of biotin, an amount sufficient for gas-phase sequencing of short peptides, was clearly detected on the modified membrane within 1 min (Scheme 1).

In active-site identification, a serious problem is the separation of the labeled and unlabeled fragments as the latter are far more abundant.[8] The use of **4** provides a strategy for distinguishing biotin-tagged peptides from nontagged peptides *via* a simple dot blot assay. Thus, we examined the combined use of the dot blot analysis method and a monomeric avidin column as an efficient means for rapid identification of GalT acceptor site. The schematic representation of our strategy is shown in Scheme 2.[9]

After photolysis, the mixture of labeled and native GalT was passed through a monomeric avidin column. The native GalT was recovered in the flow-through fraction and retained its full activity (98 ± 3%). This recovered enzyme was recycled for additional rounds of photolabeling. The biotinylated GalT was then eluted, blotted on normal PVDF membrane, and subjected

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(6) 4-Nitro-3-(trifluoromethyl)-3*H*-diazirine **2**: Nitric acid fuming (d = 1.50, 1.2 mL) was added to a stirring solution of 3-(trifluoromethyl)-3*H*-diazirine[7] (0.93, 5 mmol) in acetic anhydride (0.5 mL) at 20 °C. After silica gel column chromatography (CH_2Cl_2/hexane = 1:9), **2** was obtained as yellow oil (0.83 g, 72%): IR (film) 1530, 1350 cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.274 (d, 2H, J = 8.9 Hz), 7.37 (d, 2H, J = 8.9 Hz); EI-MS m/z 231 (M^+), 203 ($(M - N_2)^+$). Anal. Calcd for $C_8H_4F_3N_3O_2$: C, 41.57; H, 1.74; N, 18.18; F, 24.66. Found: C, 41.38; H, 1.96; N, 18.35; F, 24.38.

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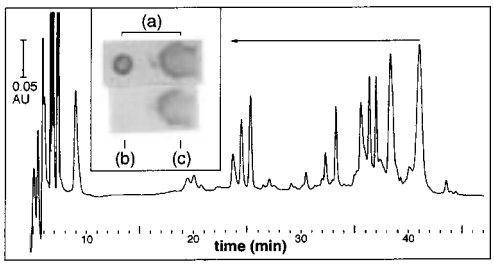

Figure 1. HPLC isolation of the biotinyl peptide from the tryptic digest of photolabeled GalT. The inset of following samples shows the chemiluminescent pattern after immobilization on **4**. (a) Both are the same sample of the peak at 41 min. (b) The left spot of a was washed with 0.01 N HCl, H_2O, 0.1 M sodium phosphate (pH 7.8) containing streptavidin (1 mg/mL) and reprobed by the chemiluminescent detection. (c) The right spot of a was similarly treated except that streptavidin was not included.

to sequential *in situ* digestion.[10] Two proteases, *Staphylococcus aureus* V8 protease and trypsin were used for the first and second digestions, respectively. The peptides released in the digestion buffers were separated by reverse phase HPLC and immobilized on the aminophenyl membrane, and their biotin content was assessed by chemiluminescent detection. Most of the peptide fragments were obtained from the second (trypsin) digestion. The peak eluting at 41 min was found to be the major biotinylated fragment as determined by the dot blot analysis (see Figure 1, inset a).

To confirm this result, a spot from this fraction was treated with streptavidin which blocked interaction with the biotin-detection reagents (inset b). An unblocked spot was positive in this test (inset c). In contrast to this, no positive test result was obtained from a nonbiotinylated control digest from native GalT.

Sequence analysis of the biotinylated fraction revealed the presence of three peptides. The sequence of the first 20 residues of the two major components corresponded to two predicted tryptic fragments of GalT S96-R129 and A98-R129. Although the initial yield of amino acid for the third fragment was very low (1 pmol), the sequence of the first 6 residues were YWLYYL corresponding to the predicted tryptic GalT fragment, Y197-R208. To distinguish the photolabeled peptide from unlabeled material, this HPLC fraction was incubated with immobilized streptavidin beads. Further sequence analysis then showed that only the minor peptide specifically adsorbed to the beads.

To confirm the results obtained with the simple dot blot analysis approach, the photolabeled mixture containing labeled and unlabeled protein was directly subjected to digestion.[11] The enzyme *Achromobacter lyticus* endoproteinase Lys-C (API) was used, since it did not produce the S96-R129 fragment that interfered with the analysis of the tryptic fragments. Biotinyl fragments from the API digest were isolated using immobilized avidin. HPLC analysis of the retained fraction showed the presence of two strong peaks, I and II (Figure 2). By contrast, these peaks were not observed in the control GalT digest in which biotinylation was blocked with an inhibitor.[5] Both peaks contained a single peptide with the sequence of the GalT fragment Y197-K230. Photolabeled amino acid residues were not observed due to the chemical instability of the cross link to the conditions of sequence analysis. To confirm the presence of biotin on both fragments, the unlabeled Y197-K230 peptide was isolated from the native GalT and shown not to bind to the immobilized streptavidin at all. These observations clearly indicate that the biotin label was incorporated on different sites of Y197-K230 sequence, as expected from simple dot blot analysis of the tryptic fragments.

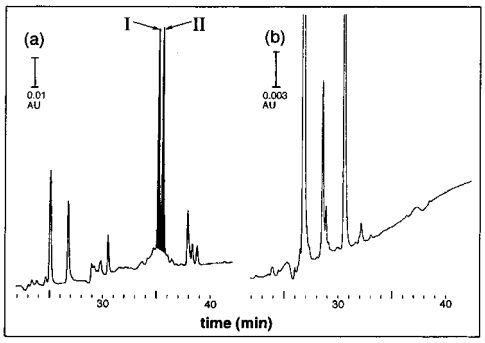

Figure 2. HPLC profiles of affinity purified API digests with monomeric avidin. (a) Peptides from the photoaffinity labeled GalT. (b) Peptides from the label-inhibiting experiment.

The previous report of trace acetylation suggests that the soluble form of bovine GalT is composed of two domains: an N-terminal acceptor substrate-binding region (275−402) and a C-terminal donor substrate-binding region (275−402).[12] An aryl azide analogue of UDP-galactose localized a large C-terminal fragment as the donor site.[13] The sequence which we have identified appears to be conserved in the N-terminal half of snail glycosyltransferase which shares the same acceptor sugar as GalT.[14] Our results thus support previous suggestions as to binding site locations and clearly demonstrate the effectiveness of our approach for rapid identification of photolabeled peptides in substrate binding sites. Such an approach, when combined with rapidly advancing molecular-cloning and protein-sequencing techniques, means that chemical identification of binding site sequences will be an attractive and challenging frontier for organic chemistry. Furthermore, combination of the present method with recently developed affinity-directed mass spectroscopy[15] will provide a powerful tool for high-resolution analysis of protein−ligand interactions.

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(9) Typical conditions: The labeled mixture of GalT (0.2 μmol)[5] was concentrated with Microcon-10 (Amicon) to ca. 100 μL at 4 °C and loaded on a monomeric avidin column (Pierce UltraLink, 200 μL, 50 mM *N*-ethylmorpholine, pH 8.0) at 4 °C. After washing, the biotinyl GalT was eluted with biotin (2 mM in 0.1 M Tris-HCl, pH 8.4). The ratio of substrate to proteases for sequential digestion was 40:1 for V8 and 100:1 for trypsin. The HPLC was performed with a reversed phase C8 column (4.6 id × 150 mm) at the linear gradient of CH_3CN from 10 to 90% in the presence of 0.1% TFA.

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(11) Photolabeled GalT (0.75 μmol) proteins were denatured (DTT; 0.4 mmol, guanidine; 68 mmol, room temperature, 1.5 h), *S*-carboxymethylated (sodium iodoacetate, 3.75 mmol, room temperature, 1 h), purified with Sephadex G50 (3 × 20 cm, 0.1 M Tris-HCl, pH 8.2), and digested with API (0.50 nmol, 2.0 mmol of urea) at 37 °C for 12 h. After gel filtration (Sephadex G-10, 4 × 18.5 cm, 0.1 M sodium phosphate, pH 7.8 (NaP)), the peptide fraction was loaded on the immobilized monomeric avidin (0.2 mL, NaP). The fraction eluted with biotin (2 mM in NaP) was analyzed by HPLC.

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