# Regioselective Labeling of Antibodies through N-Terminal Transamination

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**ABSTRACT** A convenient new method is described for the introduction of ketone groups at the N-termini of antibodies. The reaction occurs in the presence of pyridoxal-5′-phosphate under conditions mild enough to maintain antigen binding function, as confirmed by enzyme-linked immunosorbent assay. Further derivatization of these functional sites was accomplished through oxime formation, yielding well-defined antibody conjugates for a wide range of applications. The ability of the modified antibodies to bind their targets was confirmed *via* immunodot blot analysis. The generality of this method has been demonstrated on a number of monoclonal and polyclonal antibodies, all with different binding specificities.

**B**ecause of their evolvable affinity for both natural and synthetic epitopes, monoclonal antibodies (mAbs) (*1*) have attained a centrally important role as imaging agents (*2*), targeting groups for therapeutic delivery (*2*), components of diagnostic arrays (*3*), and catalysts (*4*). Many of these applications depend critically on the ability to add new function through the covalent attachment of fluorophores, anticancer drugs, enzymes, and nanoparticles (*5–7*). Typically, these groups are attached to random locations on the antibody surface through the modification of lysine, tyrosine, aspartate, and glutamate residues. Although the function of the resulting conjugates may still be intact, the nonspecific nature of the modification leads to product mixtures that may not be compatible with the intended application.

Antibodies consist of two identical light chains and two identical heavy chains held together by a series of disulfide bonds (Figure 1, panel a) (*8*). The site-selective modification of antibodies is particularly difficult to achieve due to the diverse and unpredictable set of amino acids in the hypervariable binding loops (Figure 1, panel b). As one solution to this problem, numerous applications have targeted aldehydes that can be introduced through the oxidation of carbohydrates on the Fc domain with NaIO$_4$ (*5, 9*). Alternatively, the interchain disulfides have been alkylated following reduction (*10, 11*). Recombinant techniques can be used to express the binding regions as a single polypeptide chain fused to a functional enzyme (*12, 13*).

As a simple alternative that can be used to label antibodies from virtually any source, we have applied a biomimetic transamination reaction that introduces ketone groups on the N-termini (Figure 2, panel a) (*14–19*). This reaction occurs upon exposure to pyridoxal 5′-phosphate (PLP) at 37–50 °C in buffered aqueous solution. The resulting pyruvamide derivatives can be elaborated easily through oxime formation with functionalized alkoxyamines (*20–23*). A key advantage of this strategy is its selectivity for the N-terminal amino group (*i.e.*, lysine residues do not participate) (*14*), thus affording antibody conjugates that are labeled in a limited number of locations.

Our studies toward the modification of these locations were conducted with monoclonal mouse anti-FLAG IgG (**1**) and the corresponding Fab fragments (**2**) obtained after proteolysis with papain. Samples were incubated with 10 mM PLP in pH 6.5 phosphate buffer for 18–20 h to afford ketone **4**. After removal of excess PLP *via* centrifugal ultrafiltration, the sample was labeled with fluorescent alkoxyamine **6**. Analysis by SDS-PAGE revealed fluorescent bands only in samples that had been treated with PLP (Figure 2, panel b). Higher conversion was observed when the protein was incubated with PLP at 50 °C, leading to fluorescent labeling of both the heavy (50 kDa) and light (25 kD) chains. The reaction was equiva-
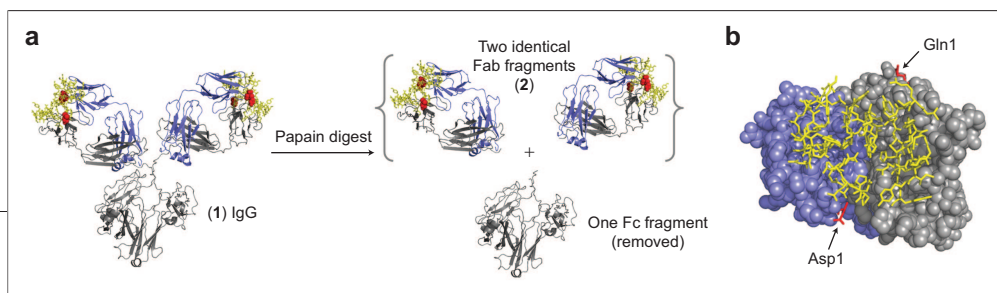
**Figure 1. Antibody structure. a)** The structures of mouse IgG (**1**) and Fab (**2**) are shown with the light chains depicted in blue and the heavy chains in gray. The amino acid residues in the hypervariable regions are rendered in yellow, and the N-termini are shown in red. Two identical Fabs are prepared *via* papain digest of IgG. **b)** Close-up view of the antigen binding site, indicating the locations of the two N-termini.

lently successful for Fab, showing modification only after treatment with PLP.

The progress of the reaction was also tracked by mass spectrometry (MS) analysis. Despite the small amount of inhomogeneity in the starting sample, the mass of Fab (**2**) could be identified at 47,848 amu, in addition to a minor species at 47,976 amu (Figure 3, panel a). After exposure to PLP, two predominant new species were observed (Figure 3, panel b). The first, at 47,803 amu, corresponded to a transamination and concomitant decarboxylation of an N-terminal aspartate, a residue that commonly occurs on the light chain of mouse IgG. (For examples of mouse IgG antibodies containing N-terminal aspartate residues on the light chain, see PDB ID 1A3R, 1IGT, 1ACY, 1BQL, 1CKO, 1DBA, 1E4W, 1F11, 1F58, and 1FB1.) We have previously observed this behavior with peptide substrates possessing this residue in this position (*14*). Light chain specificity of this mass loss was confirmed after reduction with TCEP (Supplementary Figure 2). In addition to this species, a small amount of a higher molecular weight adduct could be detected at 48,051 amu, corresponding to the addition of a single PLP molecule to the N-terminal ketone (possibly through an aldol reaction). Upon addition of benzylalkoxyamine, both of these activated species were completely converted to the singly modified products at 47,908 and 48,156 amu, respectively (Figure 3, panel c). The product resulting from oxime formation of the transaminated heavy chain terminus was observed at 47,954 amu, and the products resulting from the labeling of both N-termini were observed at 48,014 and 47,264 amu. Analogous mass changes could also be seen for the minor Fab species starting at 47,976 amu. Efforts are underway to confirm the identity of the

N-terminal PLP adduct and to suppress its formation; nonetheless, this species appears to react with alkoxyamine reagents and thus does not prevent labeling.

Although these spectra confirmed that a single modification had occurred on each chain, further evidence of the site specificity was obtained through proteolytic digest analysis. After exposure of the samples to trypsin, a single species was observed to undergo the same pattern of decarboxylation followed by oxime formation (Figure 3, panels d–f). The starting peak at 2544 $m/z$ was fragmented using tandem MS/MS to yield a peptide sequence that matched closely with other N-terminal sequences for mouse immunoglobulin light chains [Examples of protein sequences of this type can be found under NCBI accession numbers AAD34833 (identity 87%; positive 100%) AAB34860 (identity 87%; positive 100%), and PH1037 (identity 87%; positive 100%)] (Supplementary Figure 3). After treatment with PLP, MS/MS analysis of the modified peak (2499 $m/z$) indicated that the loss of 45 $m/z$ (Supplementary Figure 4) had occurred from the N-terminal aspartate residue, as seen in the b ion series (*e.g.*, the shift of the species at 328.23 $m/z$ to 283.20 $m/z$). No peaks corresponding to PLP additions were identified by MALDI TOFMS.

We were able to quantify conversion to the oxime product through the use of a previously reported poly(ethylene glycol) (PEG) alkoxyamine (**7**) (*24*). Densitometry analysis after SDS-PAGE and Coomassie staining indicated that for a transamination reaction performed at 50 °C, 47% conversion was achieved for the light chain of IgG (Figure 2, panel c). As expected, lower conversions were obtained at lower temperatures (25% at 37 °C). Only a small amount of PEG attachment was observed for the IgG heavy chain, although fluorescence data indicate

that this site can be modified with smaller alkoxyamines (see Figure 2, panel b). When the identical reaction sequence was carried out on Fab (**2**), a single new species was observed by nonreducing SDS-PAGE (20% at 37 °C and 42% at 50 °C, Figure 2, panel c). Upon exposure to DTT, the PEG-modified light chain could not be resolved from the heavy chain. No polymer conjugation was seen for the heavy chain.

Because the N-termini of IgG and Fab are in proximity to their antigen-binding regions, we sought to confirm that normal epitope recognition was not disrupted. Two complementary immunoassays were used to do this: a sandwich enzyme-linked immunosorbent assay (ELISA) and an immunodot blot. For the ELISA, modified and unmodified anti-FLAG antibody samples were first passively bound to a 96-well plate. Exposure of these wells to bacterial alkaline phosphatase (BAP) displaying the FLAG peptide was followed by incubation with anti-BAP antibodies conjugated to horseradish peroxidase (HRP). Colorimetric substrate addition yielded the same signal for antibodies activated with PLP as was obtained for their unmodified counterparts (Figure 4, panel a). We subsequently explored a dose-dependent study of IgG samples that were modified at 50 °C. If their antigen recognition capacity was diminished, a systematic loss of signal would be expected as concentrations were lowered. However, antibodies with or without exposure to PLP exhibited identical binding over the full range of concentrations investigated (Figure 4, panel b). We thus conclude that the PLP modification reaction itself has minimal effects on antigen binding capabilities.

To explore whether subsequent oxime formation perturbed antigen binding, a sandwich ELISA was also performed for Fab samples that were treated with commercially available biotin alkoxyamine **8** following reaction with PLP (Figure 4, panel c). As

before, no change in signal was observed. Biotinylated antibody samples were also used to assess antigen-binding *via* an immunodot blot. SDS-PAGE and Western blot analysis confirmed selective biotinylation of only those IgG and Fab samples that were first activated with PLP (Figure 4, panel d). Nitrocellulose membranes displaying the FLAG epitope were used to capture the antibodies through substrate binding. The blots were then probed with α-biotin mAbs conjugated to HRP to confirm the presence of the biotin group. Chemiluminescent output was observed only for samples that had been treated with PLP prior to biotinylation (Figure 4, panel e, bottom panel). Thus, the presence of a signal for biotinylated IgG and Fab, in corroboration with the ELISA data, demonstrates that these modified samples retain their ability to recognize and bind the FLAG epitope after the PLP reaction and subsequent oxime formation. For other conjugates, we anticipate that the influence on antigen binding will depend largely on the identity of the alkoxyamine that is installed.



Figure 2. Regiospecific modification of antibodies. a) Reaction scheme for the modification of 1 and 2 by PLP (3). b) SDS-PAGE analysis after fluorescent labeling of PLP-modified 1 and 2 with AlexaFluor 488 alkoxyamine (6). All samples were reduced with DTT before analysis, which separates the individual peptide chains. c) Samples of 1 and 2 were treated with PEG-alkoxyamine 7 after incubation with 3. SDS-PAGE analysis revealed the presence of PEG conjugates (indicated by the red arrows) only after PLP treatment. For 1, modification of the light chain is readily observed under reducing (+DTT) conditions (25% at 37 °C and 47% at 50 °C). Much lower levels of modification were observed for the heavy chain, preventing quantitation. For 2, a single modification (20% at 37 °C and 42% at 50 °C) is observed under nonreducing (−DTT) conditions. Under reducing conditions, the modified light chain cannot be resolved from the heavy chain.

Having shown that antigen binding is retained after modification with PLP, we wanted to confirm that this strategy could also be used to modify antibodies with other binding specificities. Using SDS-PAGE analysis after treatment with 6, fluorescent labeling of two other mAb substrates, mouse α-actin (9) and mouse α-biotin-peroxidase (10), was observed (Supplementary Figure 5). In addition, fluorescent conjugates were obtained for polyclonal goat α-mouse antibodies (11). Undoubtedly, we expect that there will be

some antibody and other protein substrates that cannot tolerate the overnight incubation steps at elevated temperatures, as this behavior is likely to vary on a protein-to-protein basis. Nonetheless, our preliminary results suggest that this method will be sufficiently broad in scope to be applied to a variety of situations.

This site-specific strategy thus provides a mild and facile approach to access well-defined antibody conjugates. Current efforts in our lab are utilizing this methodology to attach antibodies to other functional

molecules, including DNA strands and carbon nanotubes. Additional studies seek to explore the attachment of environmentally sensitive fluorophores that can report antigen binding for sensing applications.

## METHODS

**Preparation of Carbonyl-Containing mAbs and Fab.** A 600 μL Eppendorf tube was charged with a solution of IgG (1 delivered as 100 μL of a 5 mg ml⁻¹ solution in 10 mM sodium phosphate, 150 mM NaCl, pH 7.4, with 0.02% sodium azide; 1 equiv) and a solution of PLP (3 delivered as 100 μL of a 20 mM solution in
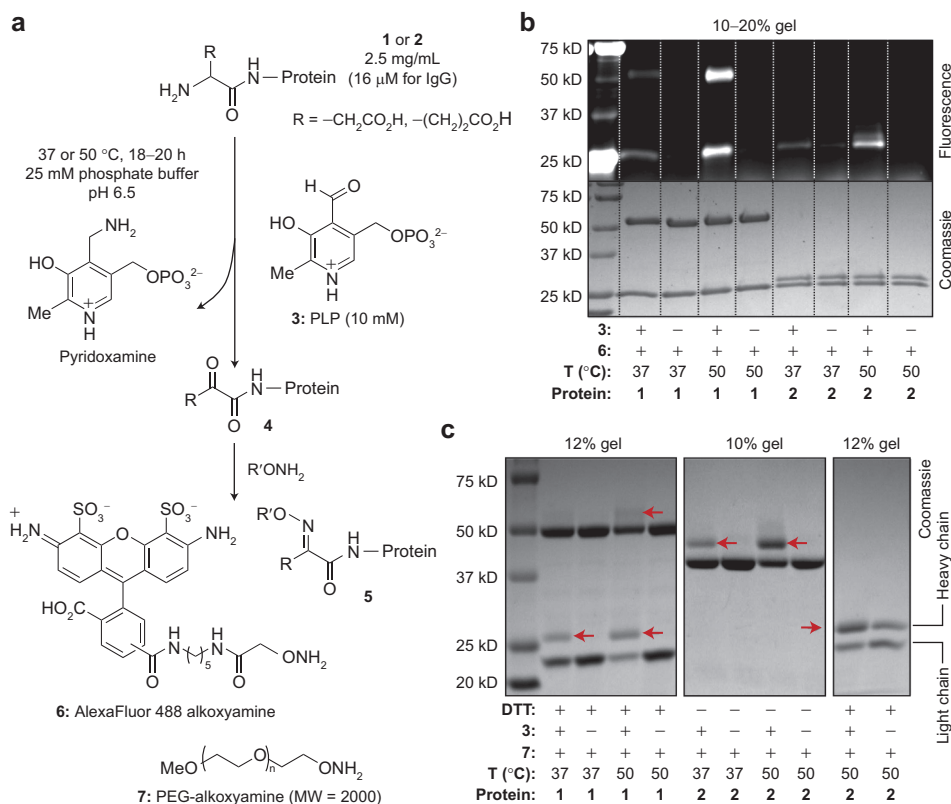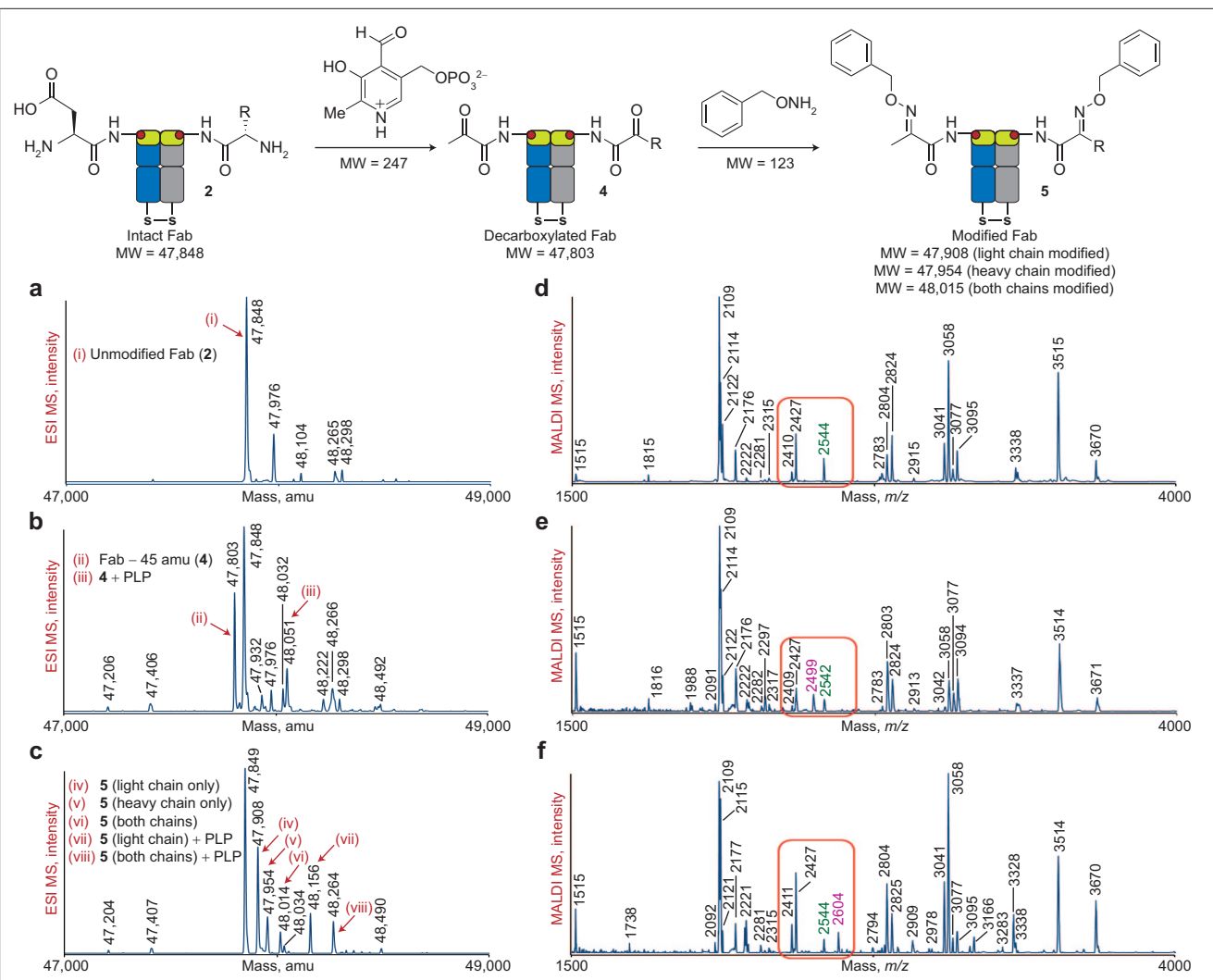
**Figure 3. MS analysis of Fab modification.** Electrospray ionization (ESI) MS spectra for a) unmodified Fab, b) Fab after exposure to PLP, and c) PLP-activated Fab further elaborated with benzylalkoxyamine. The new reaction products, along with their expected masses, are indicated pictorially above the spectra. In addition to the expected reaction products, a PLP adduct (+247) can be observed in panels b) and c). The loss of mass after PLP activation corresponds to a decarboxylation of the N-terminal aspartate residue. MALDI MS spectra for proteolytic digest of d) unmodified Fab, e) Fab after exposure to PLP, and f) PLP-activated Fab further elaborated with benzylalkoxyamine. The red box denotes the region in which the N-terminal fragment is observed. As for the intact Fab, decarboxylation e) followed by oxime formation f) is observed, though no PLP adducts are found.

25 mM phosphate buffer, pH adjusted to 6.5 with 1 M NaOH; 600 equiv). The mixture was briefly agitated to ensure proper mixing and was incubated without further agitation at 37 or 50 °C for 18−20 h. The PLP was removed from the reaction mixture via ultracentrifugation, exchanging into 10 mM phosphate buffer, pH 6.5, with 0.02% sodium azide.

**Oxime Formation.** Carbonyl-containing samples of IgG and Fab were prepared by the procedure described above. An aliquot of the purified mixture (20 μL, ~2 mg mL$^{-1}$ overall) was treated with the alkoxyamine of interest (final concentrations of 0.21−250 mM, depending on the alkoxyamine). This mixture was briefly agitated to ensure proper mixing, and was incubated at RT for 18−20 h. For more specific details regarding oxime formation, please see the Supporting Information.

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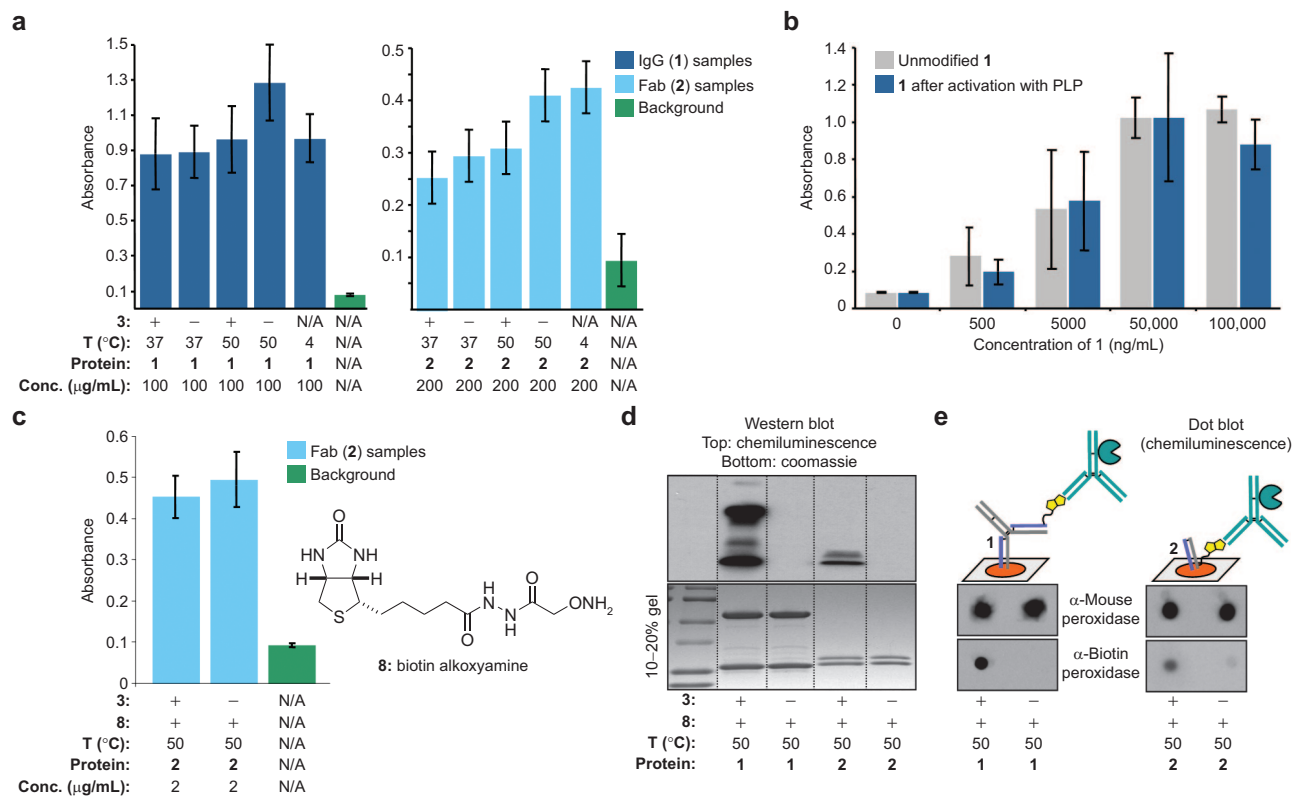
**Figure 4. Retention of antigen binding ability after modification with PLP. a)** Sandwich ELISA data for IgG (**1**) and Fab (**2**) shows little-to-no erosion of signal after modification. **b)** For samples of **1** incubated with and without PLP at 50 °C, similar ELISA signals were obtained at multiple concentrations. **c)** ELISA data for samples of **2** (with and without PLP treatment) after biotinylation with **8** (18% conversion). Minimal change in signal indicates no change in binding ability under the conditions of oxime formation with **8**. Biotin conversion is based on HABA quantitation. **d)** Western blot analysis of PLP-modified antibodies after biotinylation with **8**. Additional bands in the chemiluminescent image (lane 2) are due to impurities in the protein starting material that are below the detection limit of Coomassie staining but are observed by Western Blot. **e)** Subsequent dot-blot analysis was carried out on nitrocellulose displaying the FLAG epitope (orange). After exposure to biotinylated **1** and **2**, the membranes were probed with (i) an α-mouse-peroxidase conjugate to detect the presence of the antibodies and (ii) an α-biotin-peroxidase conjugate to confirm that they displayed the biotin group (shown schematically above the blot images).

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