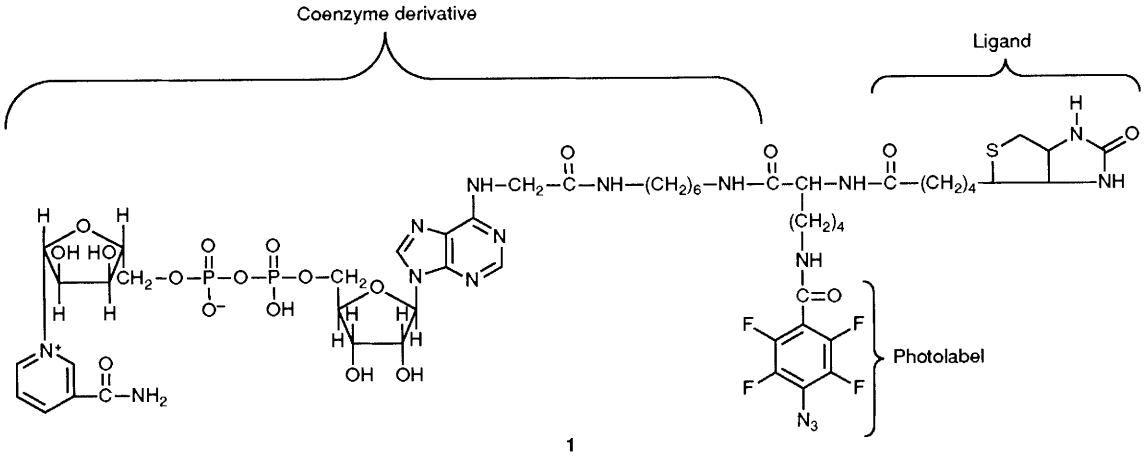
Attaching Analytes in the Proximity of the Active Site of Enzymes

Alynne I. MacLean, Tadeusz Cynkowski and Leonidas G. Bachas*

Department of Chemistry, University of Kentucky, Lexington, KY 40506, USA

A trifunctional compound, incorporating a photolabel, a coenzyme, and an analyte of interest, is synthesized and used to prepare an enzyme reagent that had been modified near its active site.

Photoaffinity labelling techniques have often been used for mapping recognition sites of biomacromolecules to reveal structure–function relationships.[1] One instance where this technique may be useful is for enzyme-linked competitive binding assays. In these assays, there is a competition between free analyte and enzyme-labelled analyte for the sites of an analyte-specific antibody or binding protein. It has been suggested that by lowering the number of analytes conjugated to the enzyme, the detection limits and sensitivities of these assays may be improved.[2] In homogeneous assays, the



1

location of the attached analyte is critical. It is anticipated that an analyte in close proximity to the active site of the enzyme will allow modulation of the enzymatic activity upon addition of the antibody or binding protein.

A trifunctional compound connecting a photolabel, an analyte, and a coenzyme to a backbone was developed. Lysine was chosen as the backbone because it contains three functional groups that allow the formation of amide bonds. A perfluorinated aryl azide was used as the photolabel because the presence of the fluorines assures a nitrene intermediate, as opposed to the less efficient dehydroazepine, which is prevalent for unsubstituted aryl azides.[3] N^{ε}-(4-azido-2,3,5,6-tetrafluorobenzoyl)-N^{α}-Boc-L-lysine† was prepared by adding triethylamine and succinimidyl 4-azido-2,3,5,6-tetrafluorobenzoate (as prepared by Keana and Cai)[4] to a suspension of N^{α}-Boc-L-lysine in dry DMF. All mixtures were stirred at room temperature under argon in darkness. Here, the solvent was evaporated under vacuum and the oily residue dissolved in ethyl acetate. This solution was repeatedly washed with 5% HCl, water and brine, and dried over magnesium sulfate. The ^{1}H NMR spectrum (400 MHz, $CDCl_3$, $SiMe_4$) displayed a series of signals [δ 1.4 (9 H, s), 1.6–1.8 (6 H, m), 3.4 (2 H, m), 4.2 (1 H, m), 5.3 (1 H, d), 7.0 (1 H, m) and 9.2 (1 H, br s)] indicative of Boc-L-lysine and the FTIR (KBr) spectrum contained a peak [ν_{max}/cm^{-1} 2128 (N_3)], which confirmed the presence of the photolabel.

Biotin, the chosen analyte, contains a carboxylic group for easy attachment to one of the amino groups of lysine. The protecting group was removed from N^{ε}-(4-azido-2,3,5,6-tetrafluorobenzoyl)-N^{α}-Boc-L-lysine by treatment with trifluoroacetic acid at room temperature.[5] Preparation of N^{α}-biotinyl-N^{ε}-(4-azido-2,3,5,6-tetrafluorobenzoyl)-L-lysine ($C_{23}H_{27}F_4N_7O_5S$) was accomplished by dissolving the previous compound in anhydrous DMSO and adding dry triethylamine followed by NHS–biotin. The solvent was evaporated under vacuum, and the residue was treated with water. The solid was collected by filtration and recrystallized from methanol–water. Peaks, showing the incorporation of biotin, were seen in the ^{1}H NMR spectrum (400 MHz, [$^2H_6$]DMSO $SiMe_4$ [δ 1.3–1.6 (6 H, m, 6-, 7- and 8-H), 2.1 (2 H, t, 9-H), 2.6 (1-H, d, 5-H_{endo}), 2.8 (1 H, d, d, 5-H_{exo}), 3.1 (1 H, br t, 2-H), 4.1 (1-H, m, 3-H), 4.3 (1 H, m, 4-H) and 6.4 (2 H, d, NH)]. The assignments of these peaks was in accord with previously published spectra.[6] The mass spectra showed the molecular ion; MS (FAB) [*m/z* 590 (M + H)$^+$].

N^6-[(6-aminohexyl)carbamoylmethyl]nicotinamide adenine dinucleotide, a derivative of NAD$^+$ (a coenzyme for G6PDH), was attached last. Lindberg *et al.* have shown that derivatization at the 6-N position still allows the coenzyme to be recognized by the enzyme.[7] The succinimidyl derivative of N^{α}-biotinyl-N^{ε}-(4-azido-2,3,5,6-tetrafluorobenzoyl)-L-lysinate was prepared by dissolving this compound in anhydrous DMSO and adding NHS followed by DCC. This solution was then added to a solution of the NAD$^+$ derivative in sodium carbonate buffer (pH 8) to yield the desired product **1** ($C_{52}H_{67}F_4N_{16}O_{19}P_2S$). After evaporation of the solvent under high vacuum, the solid was redissolved in water and the solution was passed through a Sephadex G-10 column. The ^{1}H NMR spectrum (400 MHz, D_2O; [$^2H_4$]TSP) now displayed a series of signals in the aromatic region [δ 8.2 (1 H, t, 5-H_N), 8.5 (1 H, s, 2-H_a), 8.6 (1 H, s, 8-H_a), 8.9 (1 H, d, 4-H_N), 9.2 (1 H, d, 6-H_N), 9.4 (1 H, s, 2-H_N)], which were identified as coming from the incorporated NAD$^+$ derivative. The peak assignments of 2-, 4-, 5- and 6-H_N were for protons on the nicotinamide ring; 2- and 8-H_a for protons on the adenine ring. The assignments of these peaks were based on published spectra.[8] The FTIR (KBr) spectrum [ν_{max}/cm^{-1} 2129 (N_3)] showed the photolabel to still be intact, and the MS (FAB) showed the new molecular ion [*m/z* 1435 (M − H + 2Na)$^+$].

The prepared reagent was used to modify the enzyme G6PDH in a photoaffinity reaction. Each sample was prepared by incubating G6PDH and the photoprobe at 4 °C before irradiation with a hand-held UV lamp (254 nm). Size-exclusion chromatography (Sephadex G-75) was employed to separate modified and unmodified enzyme from free unchanged reagent. Attachment of the probe was confirmed with a fluorescence study. Addition of glucose-6-phosphate, the substrate for G6PDH, allowed the reduction of any attached NAD$^+$ to NADH. When excited at 340 nm, NADH will exhibit a broad fluorescence emission band centred at approximately 450 nm. Blanks were prepared containing the same concentrations of G6PDH and glucose-6-phosphate. Samples and blanks were incubated at 4 °C overnight before the fluorescence was measured. The fluorescence signal from the samples was more than 500% higher than the blanks, confirming the presence of NADH and, therefore, the successful attachment of the analyte to the enzyme. Next, labelling of the enzyme was attempted in the presence of an excess of free NAD$^+$. This NAD$^+$ should occupy all of the active sites of G6PDH and prevent site-specific labelling by the reagent, which was shown to be the case.

This work was supported in part by a grant from the National Institutes of Health (GM 40510). A. I. M. acknow-

† *Abbreviations used*: DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; DMSO, methyl sulfoxide; G6PDH, glucose-6-phosphate dehydrogenase; NHS, *N*-hydroxysuccinimide; NAD$^+$, nicotinamide adenine dinucleotide; Boc, *tert*-butoxycarbonyl; [$^2H_4$]TSP, deuteriated trimethylsilyl propionate.

ledges support from the University of Kentucky with a Dissertation Year Fellowship.

Received, 6th May 1992; Com. 2/02340G

References

1 D. I. Schuster, W. C. Probst, G. K. Ehrich and G. Singh, *Photochem. Photobiol.*, 1989, **49**, 785; P. A. Insel, *Am. J. Hypertens.*, 1989, **2**, 112S.
2 A. I. MacLean and L. G. Bachas, *Anal. Biochem.*, 1991, **195**, 303; S. Daunert, B. R. Payne and L. G. Bachas, *Anal. Chem.*, 1989, **61**, 2160; L. G. Bachas and M. E. Meyerhoff, *Anal. Biochem.*, 1986, **156**, 223.
3 P. J. Crocker, N. Imai, K. Rajagopalan, M. A. Boggess, S. Kwiatkowski, L. D. Dwyer, T. C. Vanaman and D. S. Watt, *Bioconjugate Chem.*, 1990, **1**, 419; E. Leyva, D. Munoz and M. S. Platz, *J. Org. Chem.*, 1989, **54**, 5938; N. S. Soundararajan and M. S. Platz, *J. Org. Chem.*, 1990, **55**, 2034.
4 J. F. W. Keana and S. X. Cai, *J. Org. Chem.*, 1990, **55**, 3640.
5 B. F. W. Lundt, N. L. Johansen, A. Volund and J. Markussen, *Int. J. Pept. Protein Res.*, 1978, **12**, 258.
6 H. A. Bates and S. B. Rosenblum, *Tetrahedron*, 1985, **41**, 2331.
7 M. Lindberg, P.-O. Larsson and K. Mosbach, *Eur. J. Biochem.*, 1973, **40**, 187.
8 H. C. Lee, T. F. Walseth, G. T. Bratt, R. N. Hayes and D. L. Clapper, *J. Biol. Chem.*, 1989, **264**, 1608.