

Characterization of Polymer-Enzyme Interactions using Fluorescence

Anastassija Konash, Michael J. Cooney[✉], Bor Yann Liaw, and David M. Jameson¹
Hawaii Natural Energy Institute, School of Ocean and Earth Science and Technology.
University of Hawaii at Manoa, 1680 East-West Road, POST 109. Honolulu, HI 96822.

¹ Dept. of Cell and Molecular Biology, John A. Burns School of Medicine, University of
Hawai'i at Manoa, 1960 East-West Road, Honolulu, HI 96822.

Correspondence to: mcooney@hawaii.edu

Materials

Alcohol dehydrogenase from baker's yeast was obtained from Roche and used without further purification. Fluorescein isothiocyanate, Rhodamine isothiocyanate, Alexa 488[®] succinimidyl ester, TAMRA-X[®] and NBD (7- nitrobenzofurazan) were purchased from Molecular probes (Invitrogen). All other chemicals were of the analytical grade or higher and were purchased from Sigma-Aldrich and used as received.

Enzyme activity assay

Specific enzyme activity was measured by following the increase in the absorbance of NADH (at 340 nm) consequent to the reduction of NAD⁺ (1.5 mM) at 25°C in the presence of excess ethanol (0.2 M) and phosphate buffer (50 mM, pH 8.5). All assays were performed in triplicate and the enzyme concentration varied from 5 to 15 µg/ml.

Labeling protocols

4 mg/ml enzyme solution in carbonate buffer (50 mM) pH=8.5 was used with all the probes. The probes were weighed on microbalances and dissolved in dimethylsulfoxide (dried over molecular sieves) immediately before adding to the enzyme. Enzyme to dye

ratio in the labeling mixture depended on the probe reactivity. The best labeling results were obtained when 1:3, 1:5 and 1:10 enzyme to dye molar ratios were used for Tamra-X, NBD and Alexa 488 respectively. This incubation ratio typically resulted in a 1:1 enzyme:dye molar ratio in the final product. Labeling reaction with Alexa 488 was allowed to proceed over night at 4°C, while with TAMRA and NBD incubation was at room temperature for one hour. In all cases, the mixture was stirred continuously. After incubation, the unreacted dye was separated from the labeled enzyme on a PD-10 column (Amersham Pharmacia Biotech) using 50 mM phosphate buffer as effluent. The collected fractions were tested for the specific activity and the most active fractions were combined and concentrated using microfilters (Millipore) by centrifuging at 14000 rpm for 20 minutes. After that, labeled enzyme was dialyzed for 24 hours against 50 mM TRIS buffer, pH 7. The successful labeling of the final product was confirmed by the increase in the polarization of the dye. The degree of labeling was determined using absorption spectrum of the product following the protocol provided by manufacturer (Molecular Probes, Invitrogen, Inc.). In case of Tamra-X absorption at 535 nm the extinction coefficient of 35000 M⁻¹ cm⁻¹ was used to calculate the dye concentration¹. For NBD the extinction coefficient of 26000 M⁻¹ cm⁻¹ at 480 nm was used².

Enzyme concentration was obtained using Bradford assay (BioRad) with bovine serum gamma- globulin as a protein standard.

It was observed that the activity of the labeled enzyme decreased with the increasing degree of labeling. However, the product with enzyme:dye molar ratio of about 1:1 retained substantial activity (data not shown).

Fluorescent Microscopy

An Olympus BX51 upright epifluorescence microscope with Magnafire SP scientific grade digital camera was used to record the images. The samples were prepared immediately before the measurements. For this purposes Eastman AQ 55 dispersion in water (10% w/v) was mixed with varied amount of fluorophore and 3 µl of the mixture

was placed on the microscope slide and allowed to dry or were covered with the cover slide. The images were taken at optimal exposure time. The samples that contained only polymer were prepared to check for autofluorescence which was found negligible. In all cases, the linear dependence of the intensity of the slides on the concentration of the fluorophore was found (data not shown). Additionally the intensity of each individual slide was found linearly dependent on the exposure time (data not shown).

An Olympus FluoView™ FV1000 confocal microscope (Biological Electron Microscope Facility, Pacific Biosciences Research Center) was used to obtain the confocal images. The samples were prepared by mixing equal volumes of 10% Eastman AQ 55 and fluorophore with or without enzyme. 2 μ l of the mixture was placed on the cover slide and allowed to air dry. The image of the spot was taken with the polymer surface directed towards the objective. The slice thickness used was 1.25 μ m with 20x objective and 0.65 μ m with 40x objective. Kalman averaging was employed to reduce the noise where necessary. Stacks of confocal images were viewed and analyzed using ImageJ and Olympus proprietary software. The volume reconstruction was performed using ImageJ.

Polarization experiments.

The polarization of the fluorophore was recorded, using an ISS PC1 spectrofluorimeter (ISS Inc., Champaign, IL), in solution [3]. For solution experiments the polarization of free or enzyme-bound probe was recorded. After that, Eastman AQ 55 dispersion was added and polarization was measured again. The molar excess of the polymer over fluorophore was typically 100 fold or higher. The excitation was performed at the wavelengths as follows: 485 nm – Alexa 488, Alexa 488-labeled enzyme, NBD, NBD-labeled enzyme; 510 nm – Rhodamine, Rhodamine-labeled enzyme. The emission was viewed through an OG085 filter, which passes wavelengths above 530nm.

References:

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