Formation of protein conjugates of phosphorothioate nucleoside diphosphate beta-S

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The present study shows that the sulfur of the phosphorothioate 5'-nucleoside diphosphate beta-S adds across the olefinic double bond of the maleimide moiety of the thiol-sensitive probe, N-1 pyrene maleimide. The reaction of phosphorothioate with the maleimide moiety provided a basis for coupling the phosphorothioate nucleoside diphosphate beta-S to maleimide-derivatized bovine serum albumin. Formation of protein—nucleotide conjugates employing the thiol-reactivity of the phosphorothioate group has not hitherto been reported. Protein—nucleotide conjugates crosslinked by reaction of phosphorothioate sulfur with maleimide-derivatized protein have significant potential for ligand-mediated delivery of nucleotides.

Currently available methods for forming protein—nucleotide conjugates include ultraviolet irradiation (1), bifunctional chemicals (2) and photochemical cross-linking (3). These crosslinking methods induce changes in the bases of the nucleotide, thereby interfering with the function of the nucleotide. Also, water-soluble carbodiimides such as ethyl(dimethylaminopropyl) carbodiimide (EDC) are agents for crosslinking nucleotides to proteins (4). However, EDC lacks specificity and reacts with N1 of guanosine and also attacks thymidine (5). Moreover, coupling of proteins to nucleotides with EDC leads to protein—protein conjugates that would reduce yield of ligand—nucleotide conjugates, complicate conjugate purification and would compete with ligand—nucleotide conjugates for binding sites on the target cell membrane. The purpose of this work was to develop a practicable covalent crosslinking system between proteins and nucleotides that leaves the nucleotide bases intact and that has the potential to avoid the other aforementioned problems.

Although the sulfur of phosphorothioate nucleotides is known to possess considerable thiol character (6-11), this thiol-like reactivity has never been applied, to the best knowledge of the authors, as the basis for preparation of protein—nucleotide conjugates. In this paper we first determine the reactivity of phosphorothioate adenosine diphosphate beta-S towards maleimide with a fluorescent maleimide probe and then utilize this reactivity to form covalent conjugates of the phosphorothioate nucleoside diphosphate and a maleimide-derivatized protein.

N-1 pyrene maleimide (PM), a sulfhydryl-specific reporter molecule (12) was reacted with 5'-adenosine diphosphate beta-S (ADPS). 0.01 M ADPS (Boehringer-Mannheim) was reacted with 0.1 mM PM (Sigma Chemical Co) in 0.1 M sodium phosphate buffer (pH 8.8) with 5% acetone, at ambient temperature for 17 hours in the dark. Fluorescence emission intensity was recorded from 350—450 nm upon excitation at 330 nm.

ADPS was reacted with maleimide—modified bovine serum albumin (BSA—MAL, 20 maleimides/BSA) purchased from Pierce Chemical Co (Rockford, IL). BSA—MAL (0.035 mM) was reacted with ADPS (0.03 M) for 17 hours at room temperature, at pH 7.0 in 0.1 M sodium phosphate buffer. Controls for the (BSA—MAL)-ADPS reaction were untreated BSA—MAL and BSA—MAL in which maleimide was inactivated (BLOCKED BSA—MAL) by 2-mercaptoethanol (0.1 M) prior to the addition of ADPS. All samples were dialyzed exhaustively, diluted appropriately and absorbance of the diluted solutions was measured from 250 to 400 nm with a recording spectrophotometer.

Figure 1 shows fluorescence emission of the succinimidyl ADPS-PM adduct which indicates the reaction of maleimide and the nucleoside thiodiphosphate. The 0—0 and 0—1 band positions are characteristic of thiol adducts of PM (12). The fluorescent reaction product was also observed on a silica thin layer chromatogram (Sigma Chemical Co) by serial runs in dichloromethane, dichloromethane:methanol (5:1) and methanol, respectively (Rf = 0.7 after final run, results not shown).

Figure 2 gives the absorbance spectra of the succinimidyl adduct of ADPS and BSA—MAL (BSA—SUC—ADPS) and of blocked BSA—MAL (BLOCKED BSA—MAL), with control BSA—MAL absorbance subtracted from each. The absorbance spectrum of BSA—SUC—ADPS, with a peak at 260 nm, indicates the formation of protein—nucleotide conjugates. ADPS added to 20% of the maleimide groups in BSA—MAL, thereby crosslinking approximately 4 ADPS molecules to each BSA molecule.

The sulfur of phosphorothioate nucleotides has thiol-like character (6-11). The present study shows that the partial thiol character of the sulfur of the phosphorothioate 5'-nucleoside diphosphate beta-S enables the sulfur to add across the olefinic double bond of the maleimide moiety, thereby resulting in an increase in fluorescence of the thiol-sensitive probe, N-1 pyrene maleimide (Figure 1). The reaction of phosphorothioate with maleimide helped to provide a basis for coupling the phosphorothioate nucleoside diphosphate beta-S to maleimide-derivatized bovine serum albumin (Figure 2). The formation of protein—nucleotide conjugates employing the thiol-reactivity of the phosphorothioate group has not hitherto been reported.

In addition to the data presented here, we have found that internucleotide phosphorothioate diester and nucleoside...
Figure 1. Fluorescence emission spectrum of N-1 pyrene maleimide adduct of 5'-adenosine diphosphate beta-S. Fluorescence is measured in arbitrary units. Wavelength is in nanometers. Background fluorescence was zero. Excitation wavelength was 330 nm.

Figure 2. Absorbance of maleimide-crosslinked conjugate of adenosine diphosphate beta-S (ADPS) and bovine serum albumin (BSA). Abbreviations: BSA—SUC—ADPS, succinimidyl adduct of ADPS and BSA—MAL: blocked BSA—Mal, BSA—Mal blocked with 2-mercaptoethanol prior to addition of ADPS. The absorbance of the untreated BSA—MAL control was subtracted from the spectra.

triphosphate alpha-S yield fluorescent adducts of N-1 pyrene maleimide (13). However, the fluorescence obtained with phosphorothioate 5'-nucleoside diphosphate beta-S shown in Figure 1 was greater than that obtained with internucleotide phosphorothioate diester and much greater than obtained with nucleoside triphosphate alpha-S. Phosphorothioates thus vary in their ability to react with maleimides.

Protein—nucleotide conjugates prepared from phosphorothioate nucleoside diphosphates and maleimide-derivatized protein, as presented in this paper, have potential for ligand-targeted delivery of antisense oligonucleotides, dideoxynucleoside phosphates and genes. The biological activity of such conjugates is currently under investigation.

REFERENCES