COVALENT ATTACHMENT OF CHELATING GROUPS TO MACROMOLECULES

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This communication describes a simple procedure for covalently coupling diethylenetriaminepentaacetic acid to proteins. The technique creates hexadentate chelating sites for metal ions on proteins. A variety of metals with useful physical properties can be specifically bound to diethylenetriaminepentaacetic acid modified macromolecules. These metal-macromolecule complexes can be useful as probes in biological systems.

INTRODUCTION

The first approach to covalently coupling chelating groups to macromolecules was reported by Benisek and Richards[1], who reacted lysozyme with methyl picolinimidate to produce a bidentate chelating site. In 1974, Sundberg et.al.[2] utilized a bifunctional analog of EDTA* to create specific metal chelating sites on macromolecules. Their technique used a phenyl EDTA molecule which could be derivatized in the para position of the aromatic ring. Specifically, 1-(p-benzenediazonium) EDTA was synthesized and coupled to several proteins. Modification of proteins in this manner allows chelation of metal ions which can act as probes for various physical studies of protein structure, i.e. electron paramagnetic resonance spectroscopy, nuclear magnetic resonance spectroscopy, x-ray analysis and spectrophotometric studies. Chelation of radioactive metal ions to proteins can be useful in nuclear medicine and physiology where a protein can be used to measure a biological function. In this communication, a procedure is described which attaches the polyaminocarboxylate chelating agent DTPA to proteins using reagents common in peptide sysnthesis.

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*Abbreviations used in this communication, DTPA, diethylenetriaminepentaaccetic acid; EDTA, ethylenediaminetetraacetic acid; HSA, human serum albumin.

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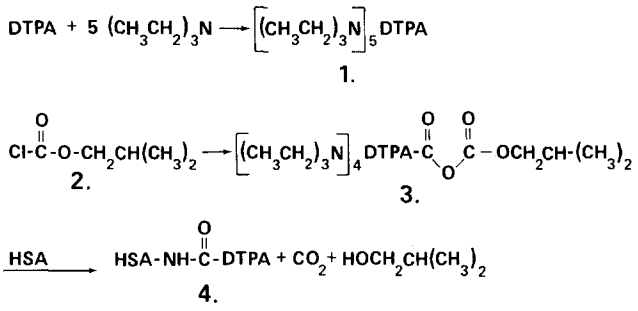

Scheme 1. Synthetic sequence for the covalent attachment of DTPA to Human Serum Albumin.

EXPERIMENTAL AND RESULTS

The synthetic sequence is outlined in Scheme 1. DTPA (0.1 gr, 0.25 mmoles, Aldrich Chem. Co.,) and triethylamine (0.125 gr, 1.25 mmoles, Aldrich Chem. Co.) were dissolved in 2 cc of H_2O with gentle heating. The solution was lyophilized to yield a glassy residue. Pentatriethylammonium DTPA (1) was dissolved in 2 cc of acetonitrile with gentle heating. The solution was then cooled in an ice bath and isobutylchloroformate (2) (0.035 gr, 0.25 mmoles, Baker Chem. Co.) was added[3]. The reaction was stirred with cooling in an ice bath for 0.5 hr during which time triethylamine hydrochloride precipitated. The resulting mixed carboxycarbonic anhydride of DTPA (3) was then added to a cooled solution of HSA (0.25 gr, Cutter Laboratories Inc.) in 20 cc of 0.1M $NaHCO_3$. As the DTPA solution was added, the pH was adjusted with 0.1M $NaHCO_3$ maintaining a pH between 7 and 8. The reaction mixture was then placed in a refrigerator at 4 °C overnight and subsequently dialyzed against 0.1M acetate buffer pH 5.0. The protein fraction was isolated by gel chromatography on Sephadex G-25. Fractions corresponding to DTPA-HSA were combined and dialyzed against 0.1M glycine-HCl buffer pH 3.5. Exhaustive dialysis and gel chromatography were necessary in order to remove excess DTPA which was not covalently attached to the protein.

Attachment of DTPA to the protein was verified by the ability of the HSA to complex indium. We labeled the modified protein with $^{113m}In^{+3}$ (available as a ^{113}Sn-^{113m}In generator, New England Nuclear) because of its usefulness in nuclear medicine. To 3 to 5 mg of protein in 1cc of glycine-HCl buffer pH 3.5 was added 1 to 2 cc of $^{113}In^{+3}$ solution. Figure 1A is the elution profile on Sephadex G-25 for the addition of $^{113m}In^{+3}$ to unmodified HSA. Under the buffer conditions chosen for this chromatography, 0.1M glycine-HCl pH 3.5, very little non-specific binding of indium to HSA was found. Free indium was first eluated in fraction 40. Unmodified HSA and $^{113m}In^{+3}$-DTPA were mixed together and similarly chromatographed (Figure 1B).

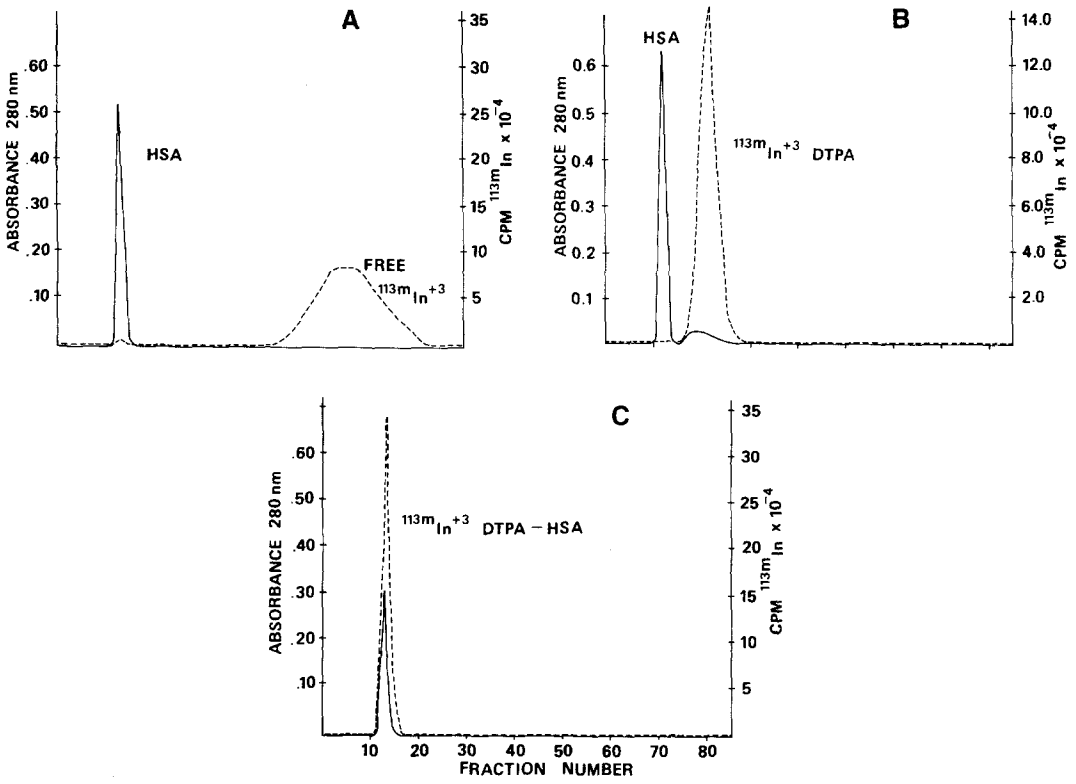

Figure 1. Sephadex G-25 chromatography of A) HSA plus $^{113m}In^{+3}$ B) HSA plus $^{113m}In^{+3}$-DTPA and C) ^{113m}In-DTPA-HSA.

Indium-113m-DTPA eluted in the second void volume with a corresponding small absorption at 280nm. In the third elution profile (Figure 1C) $^{113m}In^{+3}$-DTPA-HSA eluted as a single sharp peak in the first void volume. The labeling yield was greater than 98%.

The potential utility of chelating proteins in biological studies was demonstrated by the fact that ^{113m}In-DTPA-HSA has an *in vivo* distribution in mice nearly identical to that of ^{125}I-HSA[4] (Figure 2). Female Swiss Webster mice were injected in the tail vein with an aliquot of the corresponding radioactive protein solution. The animals were sacrificed at various time intervals by CO_2 asphyxiation and a sample of blood was taken by cardiac puncture. The organs were dissected and counted in an automatic gamma scintillation counter. The total radioactivity in the

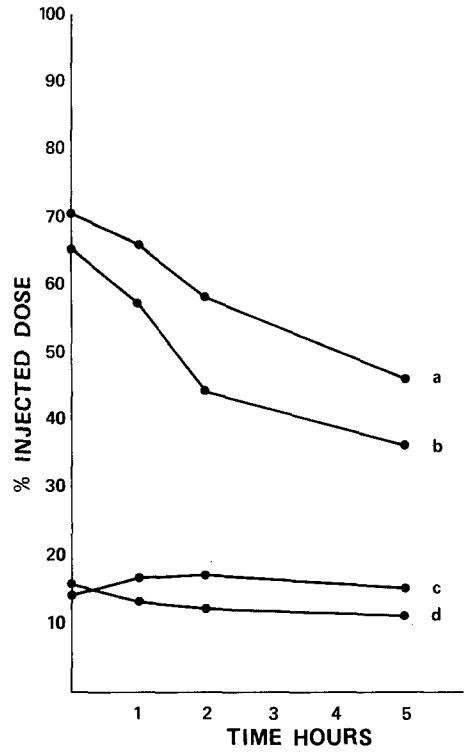

Figure 2. Biological distribution of ^{125}I-HSA and $^{113m}In^{+3}$-DTPA-HSA in mice. Percent injected dose versus time for 2a) ^{125}I-HSA in blood 2b) $^{113m}In^{+3}$-DTPA-HSA in blood 2c) $^{113m}In^{+3}$-DTPA-HSA in liver 2d) ^{125}I-HSA in liver. Each data point is the mean of five animals.

blood was calculated based on the total body weight of the animal[5]. The blood clearance rate for $^{113m}In^{+3}$-DTPA-HSA was about the same as ^{125}I-HSA. The amount of activity in the liver was identical for the two proteins. If the ^{113m}In-DTPA-HSA had been denatured during the modification and labeling procedure, the reticuloendothelial cells of the liver would have removed the protein from the blood.

DISCUSSION

The structure of In-EDTA has been postulated by employing potentiometric and polarographic studies[6]. Both nitrogen atomas are required for complex formation as well as three of the four carboxyls of EDTA. Water or halide

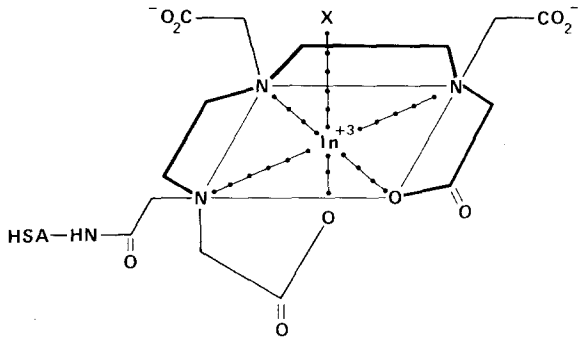

Figure 3. Possible structure for $^{113m}In^{+3}$-DTPA-HSA.

is at the sixth axial position. Assuming a similar structure for In^{+3}-DTPA, three of the five carboxyls would not be needed to chelate indium. Thus, covalent coupling of one of the carboxyls of DTPA to an amino group of a protein would not significantly alter chelation with indium. A possible structure for $^{113m}In^{+3}$-DTPA-HSA is given in Figure 3.

Direct coupling of DTPA to proteins by this route offers an alternative to the Sundberg method for preparing specific metal binding sites on macromolecules. The procedure is considerably easier to carry out and does not require synthesis of any intermediates. Transition metals such as Mn, Fe, Co, the lanthanides and the actinides would form stable complexes with DTPA modified proteins. These derivatives may prove useful in physiology and biophysical studies.

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