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Diazo Coupling of some Lanthanide Benzo-Cryptates to Proteins

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The lanthanum and praseodymium cryptate complexes of a bifunctional cryptate ligand ($2B_{NH_2}$:2:1) were diazotized in aqueous solution and then coupled to proteins. Formation of a yellowish protein solution and ultraviolet absorption around 335 nm by the solution indicated the attachment of metal cryptate to the protein through an azotyrosyl moiety. Preparation of a radioactive praseodymium cryptate and its coupling to ribonuclease-A followed by separation of the radio labelled azo-nuclease confirmed that the metal ion is resident in the cavity of the cryptate ligand even after the attachment process.

Introduction

Macrobicyclic polyether ligands (cryptands) form very stable and selective cryptate complexes with various cations (especially alkali and alkaline earth), in which the metal ion is encapsulated in the central cavity of the ligand molecule [1, 2]. The properties of these complexes may be governed by designing cryptands which incorporate special structural features. Furthermore attachment of metal complexes is a promising new area of research because metallic ions exhibit a wealth of spectroscopic and radioactive properties. The fact that diazonium compounds couple most smoothly with tyrosyl and histidyl residues of proteins [3] and that EDTA is a strong chelating agent, inspired Sundberg and coworkers to synthesize 1-(*p*-aminophenyl) EDTA, which after diazotization of its amino group was coupled to a variety of proteins [4].

The synthesis of a functionalized cryptand which on the one hand can hold the metal ion inside its cavity and on the other hand may interact, in some defined manner, with biomolecules, may in fact add new dimensions to the field of bioinorganic chemistry. The functional groups incorporated in cryptands and cryptates could then easily provide a point of covalent attachment which could be used to bind the cryptate to a macromolecule. Cryptand $2B_{NH_2}$:2:1 is the first cryptand prepared by us [5], and provides an amino group useful for attachment to a protein, an enzyme or a steroidal hormone.

Complete details of the synthesis, u.v., i.r. and 180-MHz nmr studies of some of the lanthanide complexes with $2B_{NH_2}$:2:1 have already been described elsewhere [6]. This paper presents brief accounts of diazotization and coupling of two of the lanthanide $2B_{NH_2}$:2:1 cryptates to proteins.

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Experimental

Materials

Bovine serum albumin and ribonuclease-A were obtained from Sigma Chemical Co., U.S.A. Sephadex G-25 was purchased from Pharmacia Fine Chemicals and the buffer PIPES was obtained from Calbiochem, Lajolla, CA, U.S.A. All other chemicals were either reagent grade or the best grade available. Hydrated radioactive praseodymium (Pr^{142}) metal salt was prepared from its oxide at the Radiation Laboratory of Michigan State University and was used as obtained.

Methods

The uv–visible measurements were made using a Cary-17 spectrophotometer. Radioactivity was measured using a Na(I) γ-ray scintillation detector connected to an 'Eberline' scalar-high voltage supply.

A sephadex column was prepared to separate the proteins from other small molecules. 10 gms of sephadex G-25 was weighed in a beaker and covered with water. The gel was soaked overnight. The swollen gel was then packed on a 60 $\times$ 2.5 cm column to separate the proteins. After each use, the gel was restored by washing five times with water. A 10^{-2} *M* PIPES buffer stock solution was made and the pH was adjusted to 6.50 by adding appropriate amounts of 0.1 *M* HCl. This solution was used to elute the proteins from the sephadex column.

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Diazotization of $Pr(2B_{NH_2}:2:1)(NO_3)_3$ and Coupling to Ribonuclease-A

Diazotization

Ten mg of $Pr(2B_{NH_2}:2:1)(NO_3)_3$ was dissolved in 3 ml of deionized water in a 10 ml flask. The solution was cooled to 5–10 °C in an ice bath. While stirring, 2 ml of 0.01 *M* $NaNO_2$ stock solution and a drop of 1 *M* HCl were added to the flask. The stirring at low temperature was continued for another 25 minutes.

Coupling

One hundred mg of ribonuclease-A was dissolved in 5 ml of deionized water and the solution was cooled. To the cold protein solution, diazotized salt solution was added dropwise. The stirring was continued for 15 minutes at cold temperature and another 2 hours at room temperature. A light yellow colored solution was obtained which was analyzed for azo linkage by taking the u.v. spectrum, u.v. (H_2O) λ_{max} at 276 and 330 nm.

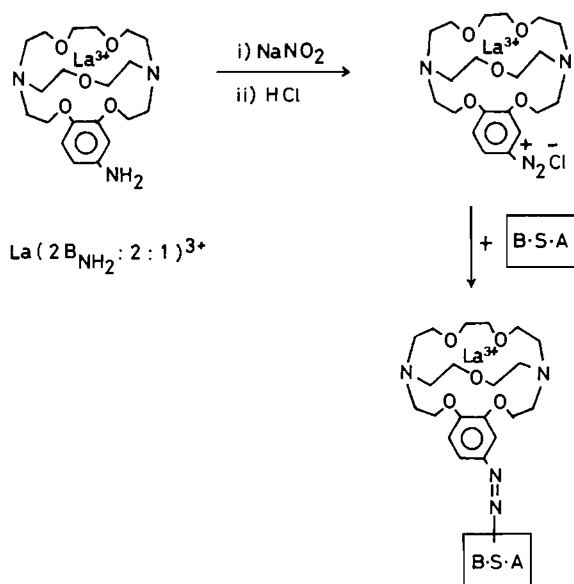
Note:

i) The same procedure was employed when radioactive praseodymium cryptate was diazotized and coupled to ribonuclease-A.

ii) A similar procedure was used for the diazotization of $La(2B_{NH_2}:2:1)(NO_3)_3$ which was coupled to bovine serum albumin.

Results and Discussion

The diazotization of $La(2B_{NH_2}:2:1)^{3+}$ in aqueous solution with sodium nitrite produced the diazotized salt which was then coupled to bovine serum albumin. As the reaction of coupling proceeded, a yellowish orange coloration of the solution was induced.



The u.v.–visible spectra of bovine serum albumin and the modified protein were taken in aqueous solution. The protein has only one absorption in the ultraviolet region with λ_{max} at 278 nm. The spectrum of the modified protein displays three absorptions with λ_{max} at 335, 275 and 270 nm. According to the literature [7], the absorption due to the monoazotyrosyl residue in proteins is found around 340 nm. It may be said with certainty that the absorption found at 335 nm in the modified bovine serum albumin is due to the transition associated with the monoazo tyrosyl chromophore in the protein. The absorption spectra of native and modified protein are displayed below in Fig. 1.

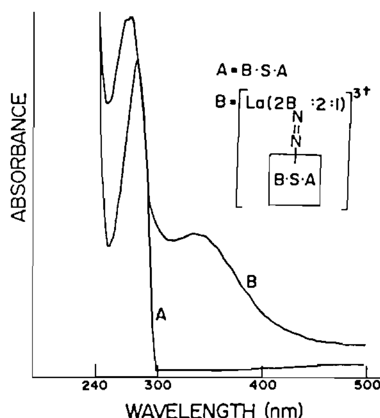


Fig. 1. Ultraviolet spectra of bovine serum albumin and lanthanum cryptate labelled azo-B.S.A.

Coupling of ribonuclease-A with $Pr(2B_{NH_2}:2:1)^{3+}$ cryptate also generated a visible colored solution. The u.v.–visible spectra of native and labelled enzyme were taken in aqueous solution. Ribonuclease-A had only one absorption in the ultraviolet region with λ_{max} at 276 nm. The spectrum of the labelled enzyme displayed absorptions at 276–278 nm and 330 nm. The absorption at 330 nm in the labelled enzyme spectrum is indicative of covalent attachment through azo bond formation.

The question of whether the metal ion remains encapsulated in the cryptate cavity after the covalent attachment to the macromolecule, was answered by preparation of a radioactive cryptate complex. The radioactive $(Pr^*(2B_{NH_2}:2:1)^{3+}$ cryptate was prepared, diazotized and coupled with ribonuclease-A.

The radio-labelled enzyme was separated from unlabelled enzyme, unreacted metal cryptate and other smaller molecules by eluting from a column of sephadex G-25. More than thirty fractions (2 ml each) were collected and analyzed for their radioactivity and absorption in the ultraviolet region. Figure 2 displays the results.

It is apparent from the figure that the fraction which carries the highest radioactivity has the highest absorption at 330 nm (azo linkage) and at

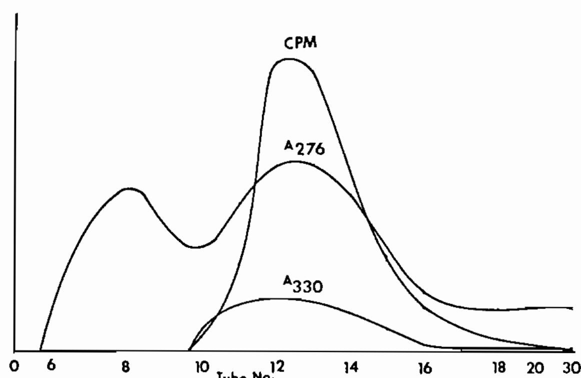


Fig. 2. Plot of u.v. absorption (at 330 nm and 276 nm) and radioactivity (cpm) vs fractions carrying the radio labelled and unlabelled ribonuclease-A.

276 nm (protein moiety). Moreover, the fractions which have only unlabelled enzyme (absorption at 276 nm only) do not carry any radioactivity. This further indicates the absence of any kind of interaction between the protein and the metal ion.

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