Acknowledgments. We wish to thank Professor D. F. DeTar for providing samples of the copolymers, which were synthesized by the *p*-nitrophenyl ester method.[14]

(14) D. F. DeTar, M. Gouge, W. Honsberg, and U. Honsberg, *J. Amer. Chem. Soc.*, **89**, 988 (1967).
(15) Recipient of Public Health Service Postdoctoral Fellowship 5-FO2-GM-41,377-02 from the National Institute of General Medical Sciences.
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Rare Earth Metal Ions as Probes of Electrostatic Binding Sites in Proteins

Sir:

One of the more important factors influencing the binding of metal ions to proteins has been suggested to be the size of the metal ion.[1] In the first-row transition series, however, where covalent interactions appear to be the dominant force, the ionic radius of the metal ion seems to bear no relationship to the strength of binding. This is shown by the following data for the binding of transition metal ions to conalbumin:[2] strength of binding, Fe(III) > Mn(II) > Co(II) > Cd(II) > Ni(II); ionic radius, 0.64, 0.80, 0.72, 0.97, and 0.69 Å, respectively.

In contrast, metal ions with closed electronic shells bind ligands through predominantly electrostatic interactions, and the strength of binding of these metal ions seems to be related to the size of the ion. Thus there is a direct relationship between the size of the ion and the strength of binding of metal ions with closed electronic shells to β-methylaspartase.[3]

Visible and ultraviolet absorption spectroscopy have been extensively employed to probe the binding sites of the first-row transition-metal ions.[4-6] Similar studies with metal ions such as calcium, which interact specifically with many proteins but which exhibit electrostatic binding, have not been feasible due to the experimental difficulty of observing electronic transitions in the vacuum ultraviolet region of the spectrum. As a result spectroscopic information about the protein binding sites of these metal ions is scarce.

The rare earth metal ions form complexes which are primarily electrostatic in nature and are analogous to those formed by the calcium ion. In contrast to the calcium ion, however, the rare earth ions exhibit sharp absorption bands in the visible and ultraviolet region of the spectrum due to Laporte forbidden f–f transitions.[7,8] These absorption bands are sensitive to both the symmetry of the complex environment and the strength of the binding of the ligand. The number of peaks observed in the absorption spectrum increases as the environment about the lanthanide ion is lowered in symmetry. The magnitude of the extinction coefficients and frequently the width of the absorption band are related to the strength of the complex formed.

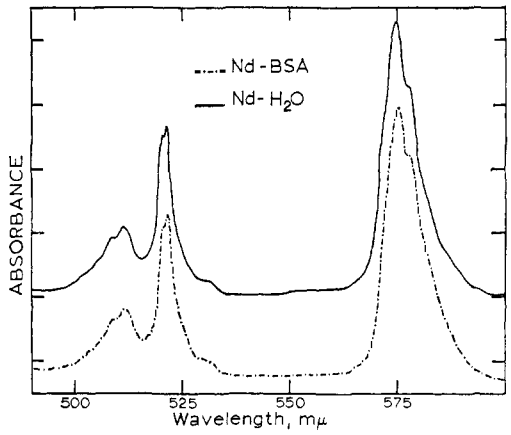

Figure 1. Absorption spectra of neodymium(III) ion (0.064 *M*) in water and in 2.11% bovine serum albumin solution (pH 5.6). These spectra were taken on a Cary 14 spectrophotometer using a 0–1.0 A slide wire. Each division corresponds to 0.1 absorbance unit.

Thus it is possible that rare earth metal ions may be used to probe calcium binding sites in proteins. This communication presents evidence that changes in the absorption spectrum of a rare earth metal ion upon binding to a protein can yield information about the protein ligands involved in complexation.

The protein which we have used in our initial experiments is bovine serum albumin (BSA). This protein was chosen because it is known to bind the calcium ion, presumably through electrostatic interactions with carboxyl groups of the protein.[9,10] The neodymium ion was the first lanthanide ion used since the ionic radii of calcium(II) and neodymium(III) are nearly identical (0.990 and 0.995 Å, respectively). Figure 1 shows the spectra obtained for the neodymium ion in water alone and in the presence of BSA at pH 5.6. These spectra are nearly identical.

The similarity of the absorption spectra of lanthanide complexes, regardless of the nature of the ligand involved, has long been a stumbling block preventing the use of these spectra in investigating complex systems. However, there are small changes in the 580- and 520-nm region of the absorption spectrum upon complexation and a difference spectrum (Figure 2) obtained using a 0–0.1 A slide wire presents a much more striking picture of these changes. The difference spectrum seems to be much more diagnostic of the type of complex formed than a simple perusal of the absorption spectrum. Because the changes seen in the 580-nm region of the spectrum are small, the sensitivity of observing complexation is limited. However, this is a region of the spectrum which is convenient for most biochemical studies.

All of the essential features of the BSA–neodymium difference spectrum have been reproduced with a variety of simple carboxylic acids. Acetate, propionate, and

(1) M. Dixon and E. C. Webb, "The Enzymes," Academic Press, New York, N. Y., 1964, p 421.
(2) A. T. Tan and R. C. Woodworth, *Biochemistry*, **8**, 3711 (1969).
(3) H. J. Bright, *ibid.*, **6**, 1191 (1967).
(4) S. K. Komatsu and R. E. Feeney, *ibid.*, **6**, 1136 (1967).
(5) K. Garbett, D. W. Darnall, I. M. Klotz, and R. J. P. Williams, *Arch. Biochem. Biophys.*, **135**, 419 (1969).
(6) G. L. Eichorn, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, **20**, 40 (1961).
(7) B. G. Wybourne, "Spectroscopic Properties of Rare Earths," Interscience, New York, N. Y., 1965.
(8) D. G. Karraker, *J. Chem. Educ.*, **47**, 424 (1970).
(9) H. A. Saroff and M. S. Lewis, *J. Phys. Chem.*, **67**, 1211 (1963).
(10) L. I. Irons and D. J. Perkins, *Biochem. J.*, **84**, 152 (1962).

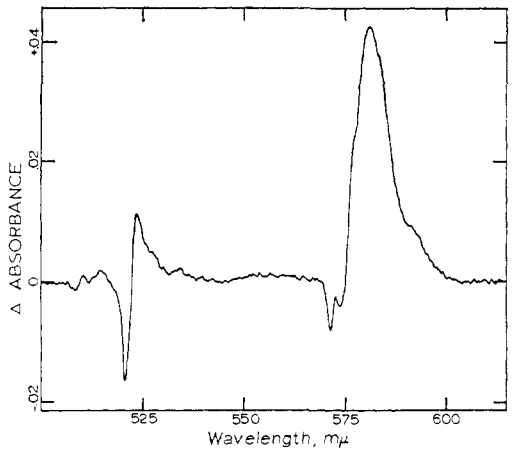

Figure 2. Difference spectrum of neodymium(III) ion in bovine serum albumin solution (sample beam) *vs.* neodymium(III) ion in water (reference beam). Concentrations of neodymium ion are identical in both cells and with those in Figure 1. This spectrum was obtained using a 0–0.1 A slide wire for the Cary 14.

maleate at pH 5.6 give nearly identical difference spectra. However, ligands which must have a different coordination symmetry, such as EDTA, or ligands which contain different coordinating groups, such as lysine and tris(hydroxymethyl)aminomethane, exhibit difference spectra in sharp contrast with the one where only simple carboxylic acid groups can bind the metal ion. These difference spectra are characterized by the appearance of new peaks in the 575-nm region as well as by change in extinction coefficients.

α-Aminocarboxylic acids such as alanine or glycine at pH 5.6 produce a difference absorption spectrum of the metal ion distinct from that of simple carboxylic acids or the other nitrogen ligands mentioned above. At this pH the amino acids are in the zwitterion forms and hence there should be little or no binding to the protonated amino group. This positive charge near the carboxyl group makes the binding of Nd^{3+} to the carboxyl weaker than with other simple carboxyl groups. This is then reflected in the spectrum observed.

When amino acids with functional side chains, which can also coordinate to neodymium(III), are used as ligands, a difference spectrum is obtained which is characteristic of the particular ligand. For example, if glutamate at pH 5.6 (where both carboxyl groups are ionized) is the ligand, a difference spectrum is obtained which is almost identical with the one in Figure 2. Additional changes are seen in the glutamate–neodymium(III) difference spectrum as the pH is adjusted to 3.0. At this pH the γ-carboxyl group is protonated, but the α-carboxyl group is still ionized. This difference spectrum at pH 3.0 is much smaller than at pH 5.6 and is nearly identical with the one obtained using alanine as a ligand. These changes reflect the difference in binding strengths of simple carboxylic acids as compared to carboxylic acids with a positive charge nearby.

If histidine at pH 5.6 is used as a ligand of neodymium(III), a difference spectrum is obtained which is again nearly identical with that of alanine. At this pH the imidazole group is protonated and hence would not be expected to bind to Nd^{3+}. At pH 7.0 the imidazole group is largely unprotonated and consequently may interact with the metal ion. We see changes in the difference spectrum at pH 7.0 over that at pH 5.6 which probably reflect this binding to the imidazole group at the higher pH. Since our protein–neodymium(III) difference spectrum was obtained at pH 5.6, it is very unlikely that protonated amino groups or imidazole groups bind the neodymium ion in the protein. Details of these and additional spectra will be presented in a later publication.

Possible ligands of neodymium in the protein are carboxyl groups, hydroxyl groups, tyrosyl hydroxyl groups, sulfhydryl groups, or the peptide linkage itself. At pH 5.6 our spectroscopic evidence with a variety of model compounds indicates that neodymium is bound to BSA only through interactions with carboxyl groups.

We would like to suggest that it should be possible to generally use rare earth metal ions to probe the binding sites of the calcium ion in proteins since the chemistry and size of these ions are very similar. Our studies indicate that much information concerning the coordinating ligands of rare earth metal ions may be gained by carefully looking at the absorption spectrum of the metal. Other methods which utilize the varied spectral and magnetic properties of the rare earth metal ions, such as circular dichroism, esr, nmr, fluorescence, etc., should likewise be applicable to the study of binding sites of these metal ions. In addition the gradual variation in size of the lanthanide ions across the series should allow a definitive test to be made of the effect of ionic radius on the binding to a protein.

We are currently investigating the possibility of replacing specifically bound calcium ion with neodymium ion in enzyme systems.

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4,6-Di-*O*-acetyl-*aldehydo*-2,3-dideoxy-D-*erythro*-*trans*-hex-2-enose. A Probable Reason for the "al" in Emil Fischer's Triacetyl Glucal[1]

Sir:

There is probably no sugar derivative more versatile in its service to carbohydrate chemists than "triacetyl glucal" (3,4,6-tri-*O*-acetyl-1,2-dideoxy-D-*arabino*-hex-1-enopyranose) (**1**).[2] Obtainable from D-glucose in a simple direct sequence,[3] it is featured frequently in studies relating to the still enthralling problem of re-

(1) We are indebted to a referee for the following comment: "The authors will be missing a wonderful opportunity if they fail to point out . . . that they may have come as close as will ever be possible to solving a scientific mystery of nearly sixty years' standing: the nature of the aldehyde-like impurity in crude glucal which induced the discoverers of this material to give it a name ending in "-al."

(2) (a) E. Fischer and C. Zach, *Sitzungsber. Kgl. Preuss. Akad. Wiss.*, **16**, 311 (1913); (b) E. Fischer, *Chem. Ber.*, **47**, 196 (1914); (c) E. Fischer, M. Bergmann, and H. Schotte, *ibid.*, **53**, 509 (1920).

(3) W. Roth and W. Pigman, *Methods Carbohyd. Chem.*, 2, 405 (1963).