Metal Chelates as Probes of Biological Systems

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There are many properties of metal ions that make them useful for solving basic problems in chemistry, biology, and medicine. These properties include the emission or absorption of photons over a large portion of the electromagnetic spectrum, radioactivity, paramagnetism, and the catalysis of chemical reactions, such as the Fenton and Haber–Weiss reactions. The utility of these properties has been enhanced by the development of metal-chelating agents that permit control over the behavior of metal ions and their localization. These chelating agents include the simple chelators such as EDTA, as well as "bifunctional chelating agents", which have a chelating group on one end and a chemically reactive functional group on the other. With the latter class of reagents, metal complexes with particular properties can be covalently attached to biological macromolecules.

Many of the most interesting applications of this technology are found in medicine. Radioactive metal ions attached by chelation to small molecules, peptides, or proteins such as monoclonal antibodies have been used clinically for diagnosis of cancer[1-6] and for studying various organs.[7-9] Metal chelates can also be used as probes in biophysical experiments. Chelate-bearing analogues of fatty acids have been employed to study model cell membranes by observing various spectroscopic signals from chelated metals.[10]

Chelates are particularly useful for energy-transfer studies using luminescent lanthanide ions such as terbium(III), and they have provided fundamental information about energy-transfer processes in solution.[11,12] Chelated lanthanides can have excited-state lifetimes longer than a millisecond in aqueous solution at room temperature. In a millisecond, an excited lanthanide can be influenced by numerous possible energy acceptors and can reveal, for example, whether an energy acceptor is buried within a macromolecule or exposed on the surface[12-16] or the nature of the electrostatic potential near the acceptor.[17]

Our work in this field dates to when one of us (C. F.M.) was a graduate student. Professor John Baldeschwieler suggested that the attachment of γ-emitting metal ions to proteins by means of "something like EDTA" could lead to interesting applictions of γ-ray perturbed angular correlations.[18-20] In designing this "something like EDTA" there were naturally many factors that were unknown. Foremost among these was whether a chelated metal at great dilution would remain chelated under physiological conditions, because of competition from metal-binding protein side chains, buffer ions, and hydroxide ions. Aminophenyl-EDTA (Figure 1, R = $C_6H_4NH_2$), prepared mainly through the efforts of Michael Sundberg, proved to have excellent stability of this sort.[1-3] In addition, its aromatic amino group could be converted into a variety of useful protein-labeling reagents. Since then, considerable work has been carried out in this lab and in others to develop new chelating agents to solve biological and biomedical problems.

The structure of a "typical" complex formed by a metal ion and an EDTA-type chelating agent is shown in Figure 1. Complexes of this general type usually have the large stability constants essential for applications as biological probes, where the probe complex may be very dilute and competing ligands are almost always present. In addition to thermodynamic stability, kinetic inertness is an important property of metal complexes. For example, in the radiopharmaceuticals used clinically, an EDTA-type complex of In(III) ion is less stable thermodynamically than other indium complexes that can form in the bloodstream. This instability is due mainly to the necessarily very low concentration of the synthetic complex but also to the relatively high con-

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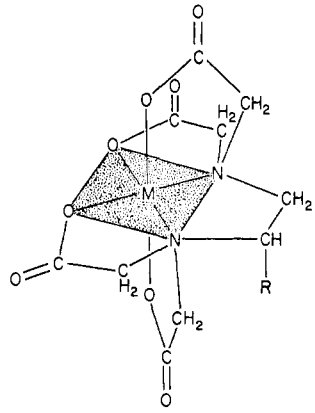

Figure 1. Example structure of a metal–EDTA chelate. The added side chain R, which usually lies equatorial to the stippled plane, is responsible for the second function of a "bifunctional" chelator.

centration of the metal-binding protein transferrin in blood serum and the high formation constant for the In(III)–transferrin complex.[21] However, if a complex is designed so that it dissociates very slowly, a metal ion can remain in the complex for prolonged periods in vivo (see Figure 4).

The remarkable versatility of EDTA and its analogues, which form stable chelates with about half of the known elements,[22,23] permits numerous applications in which the chemical or physical properties of chelated metal ions can be used in concert with biological molecules. This Account emphasizes applications involving radioactive or luminescent metal ions, which have received the most attention so far.

Chemical Syntheses and Properties

Although our original preparation of bifunctional chelators[1,2] gave useful products, the preparative scheme was tedious to carry out. The simple acylation of proteins with activated diethylenetriaminepentaacetic acid, introduced by Krejcarek and Tucker,[24] gives a rather different product[25] but is much simpler and has been more widely used.[6,7,26–28] Other routes to new bifunctional chelating agents have been devised, such as a synthesis of 1-(*p*-aminophenethyl)ethylenediaminetetraacetic acid starting from styrene.[29] Recently, we[15,30] have found that α-amino acid amides, and dipeptides, can be used as starting materials for a great variety of bifunctional chelators. As outlined in Figure 2, it is possible to make molecules with EDTA, HED3A, DTPA,[31] or any of a list of other desirable chelating

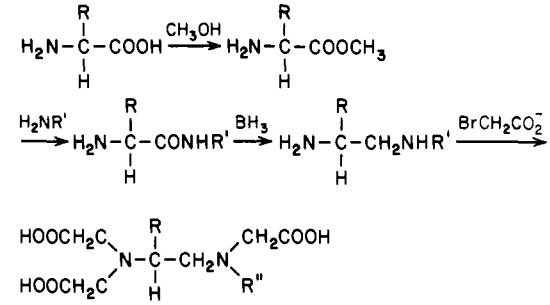

Figure 2. Conversion of α-amino acids to "bifunctional" chelators. If the side chain R of the amino acid is reactive, it must be protected (e.g., methylation of the phenolic oxygen of tyrosine). The choice of amine used in the amide-forming step leads to different chelators; for example, R′ = H leads to an EDTA group (R″ = CH_2COOH), while R′ = CH_2CH_2OH leads to an HED3A group (R″ = CH_2CH_2OH), or R′ = $CH_2CH_2NH_2$ leads to a DTPA group (R″ = $CH_2CH_2N(CH_2COOH)_2$). Beginning with nitrophenylalanine (R = $CH_2C_6H_4NO_2$) leads to a chelator with an aromatic nitro group, which after reduction to an amine (Figure 3) can be converted into many useful chemically reactive or biologically active groups.

groups. The reactive group for attachment to a biological molecule (Figure 3) can range from a simple amine or carboxyl to a haloacetamide (sulfhydryl reagent), isothiocyanate (amine reagent), or azide (photoaffinity reagent).[30,32] With aromatic amino acids as starting material, products with an aromatic ring adjacent to the chelating group are obtained. These compounds provide a useful way to excite chelating lanthanides by intramolecular energy transfer from the ring to the metal.[14,30]

The molecule schematically represented in Figure 2 has its side chain R attached to a carbon atom in the ethylene backbone of the metal-binding group. This substituent can have an important influence on the rate of dissociation of a metal ion from the chelator. Figure 4 shows the loss of ^{111}In ions from chelates to the metal-binding protein transferrin in human blood serum under physiological conditions. It is striking that the horizontal lines (indicating no loss of metal from the chelate) occur for EDTA groups with a side chain attached to a backbone carbon atom. Even a methyl side chain is large enough to show almost the full effect.[25] This is consistent with the extensive studies of Margerum and co-workers on a variety of metal–chelate systems.[33] It is also clear from Figure 4 that even the simple acylation of albumin with diethylenetriaminepentaacetic acid[24] yields a product that retains indium(III) for many hours under physiological conditions.

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(31) Abbreviations used: EDTA, ethylenediaminetetraacetic acid; $TbEDTA^-$, the Tb(III) complex of EDTA; $CoEDTA^-$, the Co(III) complex of EDTA; HED3A, *N*-(2-hydroxyethyl)ethylenediaminetriacetic acid; TbHED3A, the Tb(III) chelate of HED3A; BED2A, *N,N′*-bis(2-hydroxyethyl)ethylenediaminediacetic acid; $TbBED2A^+$, the Tb(III) chelate of BED2A; EDDA, ethylenediaminediacetic acid; en, ethylenediamine; $Co(III)EDTA^-$, the Co(III) complex of EDTA; $Cu(II)EDTA^{2-}$, the Cu(II) complex of EDTA; Cu(II)BED2A, the Cu(II) complex of BED2A; $Co(III)EDDAen^+$, the mixed complex of Co(III) with EDDA and ethylenediamine; $Tb(DPA)_3^{3-}$, the tris(dipicolinate) terbium complex; BLEDTA, a conjugate of cobalt(III) bleomycin with a bifunctional chelating agent.
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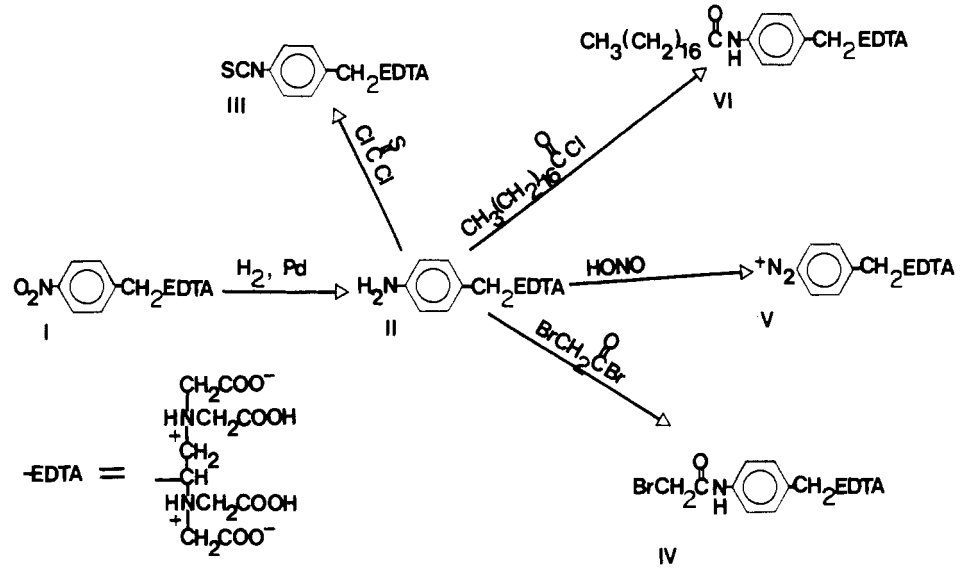

Figure 3. Examples of reactive bifunctional chelating agents, and their synthesis from *p*-nitrobenzyl-EDTA (I). The nitro group is reduced by catalytic hydrogenation[4] to produce *p*-aminobenzyl-EDTA (II). II may be converted to isothiocyanate III by treatment with thiophosgene. Treatment of II with bromoacetyl bromide converts it to *p*-(bromoacetamido)benzyl-EDTA (IV). Nitrous acid treatment of II yields the diazonium ion V. Reaction with a fatty acid chloride such as palmitoyl chloride yields an amphiphilic chelating agent such as palmitamidobenzyl EDTA (VI) (from ref 32).

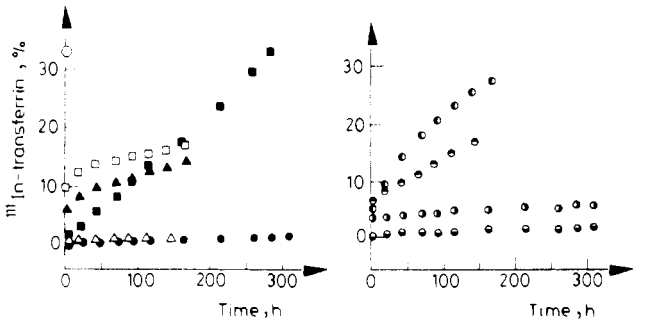

Figure 4. Time course of loss of In(III) from chelates to transferrin, determined in sterile human serum at pH 7.3 ± 0.1, 37 ± 1 °C in an air/CO_2 atmosphere:[20] (□) EDTA, (●) 1-Me-EDTA, (△) 1-(*p*-carbomethoxybenzyl)-EDTA, (○) EDTA mono-*n*-butylamide, (■) DTPA, (▲) DTPA mono-*n*-butylamide, (◓) DTPA-HSA (HSA = human serum albumin), (◐) DTPA-HSA (0.01 M glycine), (◒) phenyl-EDTA-HSA, (◑)) benzyl-EDTA-HSA (from ref 25).

This result has been borne out in several in vivo studies.[6,7,26,28,34]

BLEDTA, a Tumor-Localizing Molecule

So far, the primary use of bifunctional chelating agents has been for preparing radiopharmaceuticals. These radioactive compounds bear short-lived γ-emitting nuclides that are used in medical diagnosis. For example, the anticancer drug bleomycin has the unusual property of selectively accumulating in some types of cancer cells, and radioactive bleomycin can be used to radiolabel and thus locate cancerous areas in the body.[35-37] Bleomycin is able to bind a number of metal ions, but only its complex with kinetically inert cobalt(III) remains associated long enough for use as an in vivo diagnostic reagent.[36,38-42] It occurred to David Goodwin and C.F.M. that attaching a metal chelator to a nonessential site on bleomycin might permit the use of short-lived radionuclides such as ^{111}In (half-life 2.8 days) to make radiopharmaceuticals. In principle, these would be safer than radiopharmaceuticals based on ^{57}Co (half-life 270 days). The synthetic plan that was ultimately developed in this laboratory[4] is shown in Figure 5. We noted that the stable cobalt(III) complex of bleomycin was selectively taken up by tumor cells.[37] It also permitted chemical modification of the dimethyl(γ-aminopropyl)sulfonium group (the "terminal amine") shown on the right side of Figure 5. This group was a promising site for a variety of modifications, since naturally occurring bleomycins have different terminal amine groups but show similar biological properties.[43] However, attachment of a chelator (M_r 400) to a relatively small molecule such as cobalt(III) bleomycin (M_r 1500) seemed to have a significant chance of yielding a product with no affinity for cancer cells.

As shown in Figure 5, BLEDTA was prepared by adding nonradioactive cobalt(II) to bleomycin A_2 and allowing it to air-oxidize to stable cobalt(III) bleomycin A_2. The dimethylsulfonium group was then demethylated and alkylated at the sulfur with a bifunctional chelator.[4] BLEDTA contains a cobalt(III) ion coordinated to bleomycin side chains and a metal-free EDTA group attached to the terminal amine region. Because of the kinetic inertness of Co(III), this situation persists indefinitely under ordinary conditions. BLEDTA can be radiolabeled quickly with a metal ion such as the

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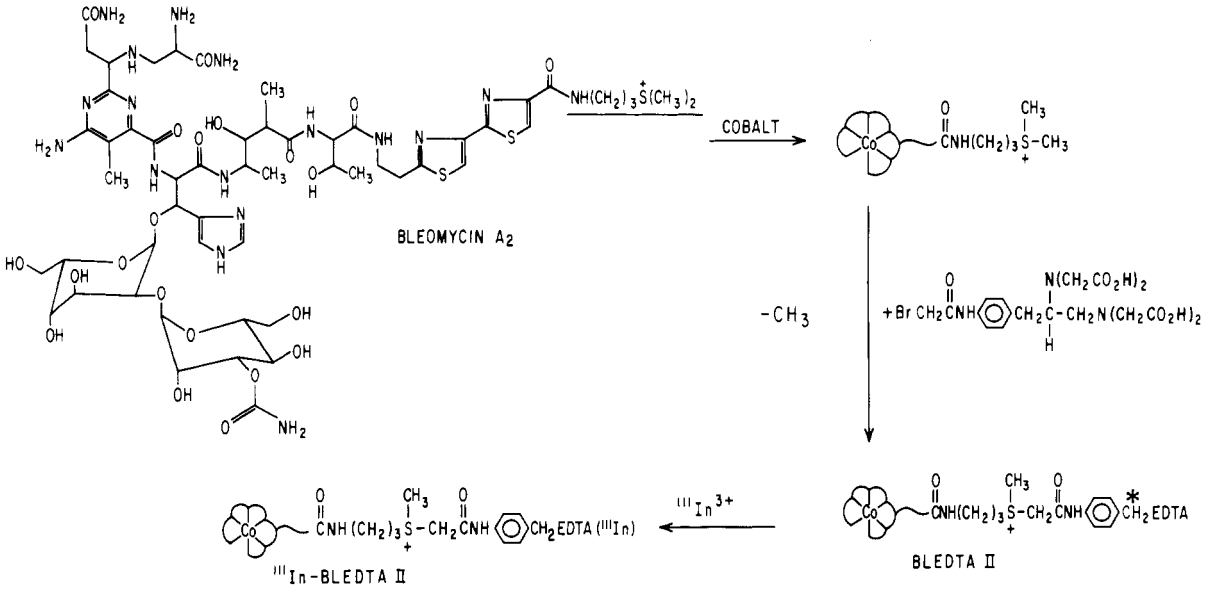

Figure 5. Synthesis of BLEDTA II. Bleomycin A2 is shown in detail; the terminal amine is underlined. After the cobalt(III) bleomycin A2 complex (shown schematically with only the terminal amine portion shown in detail) is isolated, it is demethylated to cobalt(III) bleomycin A2DM and then alkylated with L-(*p*-(bromoacetamido)benzyl)EDTA to give BLEDTA II (from ref 4).

clinically useful $^{111}In^{3+}$, and it has been found to be valuable for diagnosis of cancer in humans.[4,5] This example shows that in favorable cases a group as large as bifunctional chelator can be conjugated to a relatively small biological molecule without seriously altering the desirable properties of either.

Chelators Linked to Antibodies

With the availability of monoclonal antibodies, which bind with great selectivity to biological molecules,[44,45] the biomedical applications of bifunctional chelating agents were significantly enhanced. These antibodies can be used in vitro for analysis of hormones and other biological compounds, or they can be used in vivo for diagnosis and treatment of disease.[46-49] Attached metal chelates have served as fluorescent or radioactive labels[6,7,26,28,50] and may provide radiotherapy in the future.

The attachment of chelating agents to antibodies is now a routine procedure in our laboratory. With a new antibody, we usually use an isothiocyanate (III in Figure 3) and a bromoacetamide (IV in Figure 3) in separate reactions, to see which will work better. With antibody concentrations greater than 10 $\mu g/\mu L$, products containing on the average more than one chelator/antibody may easily be prepared and then purified by dialysis or gel filtration. The resulting antibody-bound chelators ($>10^{-5}$ M) may be quickly labeled with metal ions, after which the products can be diluted as desired.[51] Straightforward titrations with standarized metal solutions (radioactive or luminescent) are used to count the number of protein-bound chelators. Although this work is still at an early stage, we and others[6,7,26,28] have found no significant problems in producing biologically active products.

Probes for Lanthanide Energy Transfer

A fairly new and fruitful area of basic biophysical research utilizes energy transfer to and from chelated metal ions. Luminescent lanthanide ions (e.g., Tb(III) and Eu(III)) are well-suited for energy transfer due to the isotropic character of their emissions. The quantum states of the lanthanide f electrons, which are responsible for their spectral properties, are relatively insensitive to the environment, so it is believed that lanthanides emit radiation in all directions with no preference for any molecular frame of reference.[52] As a result, the problem of relative orientation of donor and acceptor, which can introduce considerable ambiguity into energy transfer experiments designed to measure fixed distances between donors and acceptors, is minimized. Another advantage is that the long (ca. 1 ms) luminescent lifetimes of chelated lanthanides make it possible to quantitate energy transfer without interference from scattering or any organic fluorophores which may be present.

In 1976 we discovered that when trivalent terbium or europium was added to a solution of a chelate-tagged protein, luminescence from the chelated lanthanide ions was easily observable.[53] This enhanced luminescence is caused by energy transfer to the chelated metal from the adjacent aromatic ring.[54] A research group led by Soini[50] has used this basic observation in developing an immunoassay procedure. With the development of new coupling chemistry for binding metal ions to macromolecules, more applications of this type can be expected.

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Energy Transfer in the Rapid-Diffusion Limit

To date, most of the energy-transfer studies using synthetic metal chelates have involved diffusion-enhanced energy transfer from *freely diffusing* lanthanide chelates. C.F.M. was introduced to this type of energy transfer in Lubert Stryer's laboratory, where experiments were being carried out with dipicolinate complexes of Tb(III) as donors and dyes as acceptors.[13] Because of its millisecond excited-state lifetime, an excited terbium complex in solution can diffuse thousands of nanometers and encounter many possible energy acceptors before it decays. In this extreme situation the rate of energy transfer does not depend on diffusion coefficients but depends strongly on the distance of closest approach between donor and acceptor.[12,13,15] Measuring energy transfer provides a means of determining whether a chromophore is exposed on the surface or buried within a biological macromolecule or membrane.

Energy transfer in the rapid-diffusion limit obeys pseudo-first-order kinetics. After a pulse of exciting light, the population of excited donors decays exponentially with time according to

$$\frac{-\mathrm{d[Tb^*]}}{\mathrm{d}t} = \frac{1}{\tau}[\mathrm{Tb^*}] = \left\{\frac{1}{\tau_0} + k_2[\mathrm{A}]\right\}[\mathrm{Tb^*}] \quad (1)$$

where [Tb*] is the molar concentration of excited donors, τ is the observed donor lifetime in the presence of acceptor, τ_0 is the donor lifetime in the absence of acceptor, [A] is the molar concentration of acceptors, and k_2 (M^{-1} s^{-1}) is the second-order rate constant for energy transfer. Measuring τ, τ_0, and [A] permits determination of k_2. All donors in the system will have the same value of k_2 only if each is influenced by many potential acceptors during its lifetime. In aqueous solution at room temperature, for a donor with a millisecond lifetime this condition is satisfied adequately for acceptor concentrations greater than 10^{-6} M.[13,15]

A useful feature of the chelating agents described above (Figure 2) is that simple variations in synthesis can yield UV-absorbing aromatic chromophores[30] that greatly enhance the lanthanide luminescence by intramolecular energy transfer or chelates that form metal complexes with different net electric charges.

Electrostatic Effects

In the course of early experiments with diffusion-enhanced energy transfer, it occurred to us that use of electrically neutral chelates, rather than complexes like $Tb(DPA)_3^{3-}$ and $TbEDTA^-$, could give more definitive information about chromophore accessibility by eliminating interference from electrostatic effects. On the other hand, the use of neutral and charged donors in parallel experiments could yield information about the electrostatic environment of the acceptor. The basic idea was similar to the use of ionic strength dependence in studies of ionic chemical reactions. The new twist was that the probe ions did not interact chemically with the acceptors and that the neutral probe (TbHED3A), containing the same donor in a complex whose size and shape were almost identical with the charged probe ($TbEDTA^-$), could serve to give an intrinsic rate constant in the absence of electrostatic effects.

We decided to test these ideas quantitatively using transition-metal complexes as acceptors, because these could be accurately modeled as hard spheres with central charges. In the rapid diffusion limit, energy transfer should be sensitive to the equilibrium radial distribution of acceptors about the average donor. For spherical ions of low charge, theoretical equations for equlibrium distributions are available, which are in accord both with modern statistical mechanical calculations and with experimental data on salt solutions.[55]

Consider energy transfer between two metal chelates that occurs through close contact between donor and acceptor, i.e., a collisional mechanism. If we compare energy transfer from a neutral donor to that for a charged donor, their rate constants should be related by a simple Boltzmann factor:

$$k_{ex} = k_0 \exp[-w(a)/k_B T] \quad (2)$$

Here k_{ex} is the rate constant for energy transfer from the charged donor, k_0 is the corresponding constant for the neutral donor, k_B is Boltzmann's constant, and T is the absolute temperature.[11] The quantity $w(a)$ is the potential of mean force between donor and acceptor when they are in contact at the distance a. With use of the Debye–Hückel theory $w(a)$ is given by

$$w(a) = \frac{Z_a Z_d e^2}{\epsilon a(1 + \kappa a)} \quad (3)$$

where Z_a is the acceptor charge, Z_d is the donor charge, e is the electron charge, ϵ is the dielectric constant of the medium, and κ is the inverse Debye length given by $\kappa = 3.3I^{1/2}$ nm^{-1} for water at 298 K. This expression is based on a simple mathematical model of charged spheres in a continuous medium. All quantities in the equations can be determined independently, and there are no adjustable parameters.

When rates of energy transfer from TbHED3A and $TbEDTA^-$ to $Co(III)EDTA^-$ and $Cu(II)EDTA^{2-}$ were measured,[11] the results were predicted accurately by these equations over a wide range of salt concentrations (Figure 7). Moreover the equations for the long-range dipole–dipole energy transfer could not be used to fit the data. Recent experiments with neutral Cu(II)-BED2A, and with positively charged $Co(III)EDDAen^{1+}$ as acceptors have provided further support for the collisional mechanism. Recently[17] we synthesized $TbBED2A^+$ to complete a triad of electrostatic probes (Figure 6). Experiments with this probe and variously charged acceptors were also in agreement with eq 2. These show clearly that, for these systems, energy transfer is dominated by short-range processes such as the exchange interaction[56] rather than the long-range dipole–dipole interaction. Indeed, for the example in Figure 7, dipole–dipole energy transfer is calculated to account for only 5% of the observed rate.

To extend this technique to biological macromolecules, we chose a protein whose electrostatic properties were well-known. Sperm whale met(aquo)myoglobin has a visible absorption due to its heme group that makes it a good energy acceptor (Figure 8). Furthermore its structure is known, and its electrostatic properties have been the subject of extensive study (e.g., ref 57 and 58). By changing the pH from 6 to 8.2, it is

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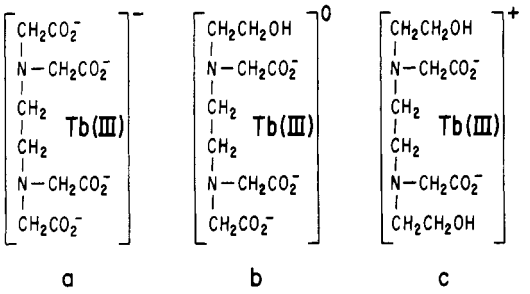

Figure 6. Structures of the series of Tb(III) chelates used as energy-transfer probes to determine electrostatic effects: (a) $TbEDTA^-$, (b) TbHE3A, (c) $TbBED2A^+$. These complexes are of similar size and shape, differing primarily in their net electric charge (from ref 17).

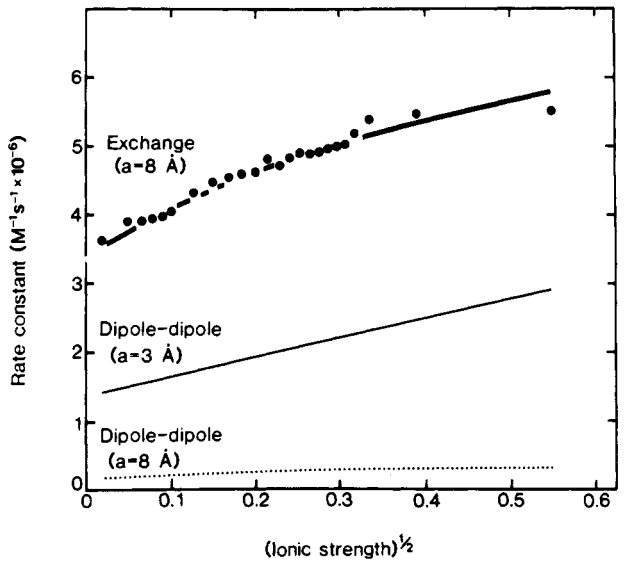

Figure 7. Ionic strength dependence of rate constants for energy transfer in the rapid-diffusion limit from uninegative $TbEDTA^-$ to uninegative $Co(III)EDTA^-$. The observed rate constants (solid circles) fit the curve (upper solid line) calculated for the exchange interaction (eq 2 and 3) with $a = 0.8$ nm and $k_0 = 8.35 \times 10^8$ M^{-1} s^{-1}. The middle curve is calculated for dipole–dipole transfer (which depends on the inverse sixth power of the donor–acceptor separation) with $a = 0.3$ nm and the lower one for $a = 0.8$ nm (from ref 11).

possible to vary the net charge of myoglobin from about +5 to zero.

Figure 9 shows the rate constants for energy transfer from each of the probes in Figure 6 to met(aquo)-myoglobin, as a function of pH. The magnitudes of the rate constants[17] are consistent with the accessibility of the protein-bound heme group as revealed by the crystallographic model;[59] both indicate that the chelate can contact one edge of the heme. The most striking feature of Figure 9 is that the rate constants for all three probes converge near the isoionic point of myoglobin.[57] Also, the points for the positive and negative probes are symmetrically disposed about the points for the neutral probe, indicating that both charged probes are responding to the same effects with increasing pH. Assuming that myoglobin behaves as a uniformly charged sphere, we compared calculated rate constants, based on the known charge of myoglobin at each pH value, with the observed ones. In doing so, we could assume

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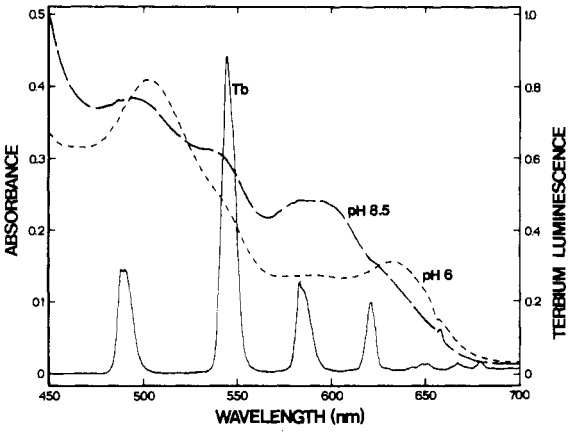

Figure 8. Spectral overlap of Tb(III) emission and myoglobin absorbance: (---) absorbance of met(aquo)myoglobin, pH 6.0; (– –) absorbance of meta(aquo)myoglobin at pH 8.2; (—), corrected emission spectrum of TbHED3A, excited at 352 nm (from ref 17).

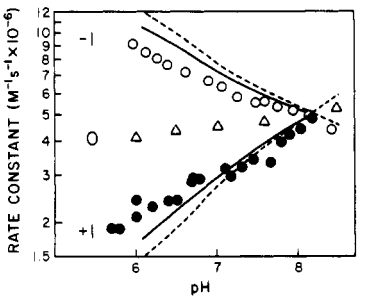

Figure 9. Semilogarithmic plot of second-order rate constants for energy transfer from terbium chelates to met(aquo)myoglobin as a function pH. The donors were as follows: (○) $TbEDTA^-$, (●) $TbBED2A^+$, (△) TbHED3A. The solid curves were calculated for dipolar energy transfer as described in ref 17. The dashed lines were calculated for collisional (exchange) energy transfer (from ref 17).

either that a collisional mechanism was predominant or that a dipolar mechanism dependent on the inverse sixth power of donor–acceptor separation was responsible for the observed energy transfer. Figure 9 shows the theoretical curves derived from these calculations. The calculated values, especially for the dipolar mechanism, closely parallel the experimental results. So in this case it is possible to use terbium chelates to measure the accessibility of a chromophore and also the charge of the macromolecule that contains it.

RNA Polymerase Inhibitors

Diffusion-enhanced energy transfer can be used to determine the accessibility of chromophoric sites in macromolecules where the overall structure and the location of the binding site are uncertain. We have used this approach in our study of the enzyme RNA polymerase. The chromophoric inhibitors rifamycin and cibacron blue have been used as probes to investigate the mechanism of RNA polymerase, and we were curious to know whether the chromophoric portions are exposed on the surface when the inhibitors are bound to the enzyme. To answer this question, we measured energy transfer rate constants (k_2) for the inhibitors using as a donor the spherical, electrically neutral chelate TbHED3A. The experiment was carried out first in the presence and then in the absence of RNA polymerase. Figure 10 shows how the ratio of rate constants for bound and free rifamycin is expected to vary as the

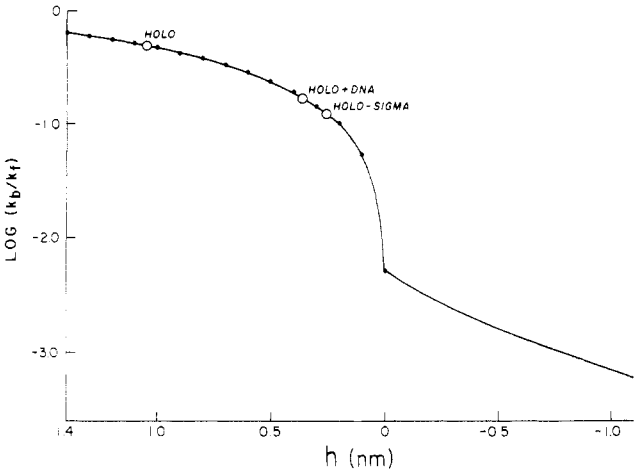

Figure 10. Effect of the σ subunit and DNA on the accessibility to TbHED3A of rifamycin bound on RNA polymerase. The curve represents the logarithm of the ratio of rate constants for energy transfer to the bound rifamycin (k_b) and the free rifamycin (k_f) that would be expected for a sphere the size of rifamycin embedded in a surface, and extending a distance h above the surface. The small filled circles are theoretical points. The open circles represent the experimental results for the holoenzyme (i.e., with the σ subunit bound), for the core enzyme (without the σ subunit), and for holoenzyme bound to DNA (poly[d(A-T)]). These data indicate that either removal of σ, or addition of DNA to the holoenzyme, results in the rifamycin binding site becoming less exposed to small molecules in solution.[15,16]

bound rifamycin becomes buried in a flat surface (representing the enzyme). It was found that k_2 for rifamycin dropped about 50% when it became bound to RNA polymerase,[15] consistent with the chromophore being only half-buried. Cibacron blue, on the other hand, appears completely buried when bound to the enzyme. Under conditions where about 90% of the cibacron was bound (it binds much less tightly to RNA polymerase than does rifamycin), the small amount of free cibacron accounted for practically all the observed energy transfer.

Since rifamycin does not block the normal DNA binding function of RNA polymerase, it was possible to determine the effect of bound DNA on the accessiblity of enzyme-bound rifamycin to the terbium probe. In addition, RNA polymerase exists in two different forms at different stages of transcription: "holo" (with all subunits bound together) and "core" (with one polypeptide subunit, called σ, dissociated). So we were also able to look at the effect of the σ subunit on rifamycin accessibility. The results are shown schematically in Figure 10; binding DNA to the holoenzyme reduces k_2 by an additional factor of between 2 and 3, and dissociation of σ produces a similar decrease in accessibility of the inhibitor.[16]

An interesting feature of Figure 10 is the steepness of the theoretical curve near $h = 0$. The rate of energy transfer is clearly a very sensitive indicator of whether or not the energy acceptor can contact chelate-sized molecules in solution, due to the large contribution of the exchange mechanism when collision is possible.

Other examples of this sort of accessibility study have included studies on the trans-membrane location of the visual pigment retinal in the proteins rhodopsin and bacteriorhodopsin,[60,61] the metal-binding sites of the iron transport protein transferrin,[14] and more recently determination of the accessibility of various dyes and antibiotics (e.g., ethidium, acridine orange, bleomycins) bound to DNA.[62]

We have also used diffusion-enhanced energy transfer to probe the electrostatic properties of DNA. DNA has a strong electric field due to the high linear density of phosphate groups along the polymer backbone. We have found that the terbium probes do not bind to DNA to any measurable extent and that large differences (over 3 orders of magnitude in some cases) can be observed between rate constants for energy transfer from probes of opposite charges (e.g., $TbBED2A^+$ and $TbEDTA^-$) to acceptors (e.g., ethidium) bound to double-stranded DNA.[62] This enables us to estimate the electrostatic potential at the surface of the DNA–acceptor complex.

Analogous applications of chelated lanthanides have been based on their paramagnetic properties. For example, experiments with cytochromes have used Dy(III) chelates to perturb the EPR signals from the prosthetic group metal ions.[63] Recently we have found that Gd(III) chelates with the series of ligands shown in Figure 6 selectively broaden the proton NMR signals of cobalt(III) bleomycins. However, weak binding of the probe to the bleomycin may have an important effect in this case.[64]

Concluding Remarks

The work with chelating agents to date has laid much of the fundamental groundwork necessary for expanded applications in biology and medicine. Although much more remains to be learned, many of the basic questions regarding the properties of metal chelate–macromolecule conjugates have been answered.

Bifunctional chelating agents have proven to be uniquely useful tools for linking metal ions to desirable targets. In designing such reagents, there is great flexibility of choice regarding the structure and reactivity of the final product. Many different chelators have been prepared, and many more can be developed by straightforward variations of the general synthetic strategy.

An informed choice for a given application requires not only knowledge of thermodynamic stability constants of metal chelates but also knowledge of the kinetics of their association and dissociation.[25,33] For example, note in Figure 4 that the loss of indium is faster from DTPA ($K_s \simeq 10^{29}$) than from EDTA ($K_s \simeq 10^{25}$) than from 1-Me-EDTA ($K_s \simeq 10^{26}$). Our approach to the complex problem of metal ions in biological systems has been to select chelators that react with a variety of metal ions to form products that are kinetically inert as well as thermodynamically stable. This permits their use in new situations without much concern for decomposition.

One of the most active areas of application of bi-

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functional chelating agents will probably continue to be clinical use of radioactive metal ions bound to proteins, particularly monoclonal antibodies. The ability to localize metal complexes at particular sites is also likely to have other uses in biochemistry and medicine.

The applications of unattached chelates, especially in the study of fundamental physical properties of biological molecules and supramolecular complexes, are just beginning to be explored. The existence of a diverse collection of chelating agents suggests a number of experiments in which the physical properties of chelated metal ions can be used to probe biological systems.

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Differential Geometry and Protein Folding

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Proteins are biologically produced, specific-sequence copolymers whose monomer units are the 20 naturally occurring amino acids. They are distinguished from synthetic polymers by their ability to undergo a reversible thermally or chemically induced transition from an unfolded, biologically inactive form to a native form that executes, at most, small fluctuations about a well-defined conformation and that exhibits full biological activity.[1] This "native" conformation is so sharply defined that many proteins can be crystallized and their structures elucidated by X-ray diffraction methods. Both experimental evidence and theoretical considerations suggest that this refolding is not the result of a random search by the molecule but rather is an efficient directed process that depends on a sequence of nucleation steps for the rapid attainment of the correct conformation. In this picture, the imposition of renaturing conditions leads to the formation of regions of structure whose frequency and/or amplitude of fluctuation are substantially less than those of the remainder of the chain. These nuclei either interact with one another or cause other regions of the chain to interact in a manner that leads to correct folding in a minimal number of steps.

This mechanism implies the existence of a hierarchy of time and length scales that characterize the folding process. It further suggests that these two scales are correlated, i.e., those structures that form in the shortest times are those characterized by the shortest length scale, and larger structures are formed at later times in the folding process.

Inspection of X-ray structures of proteins indeed reveals the presence of certain characteristic structures. These characteristic structural features of proteins[2] can be classified as either of undetermined length scale or defined on a particular scale. Right-handed α-helices and extended strands are not limited in their length by any structural factors internal to themselves. Bends, on the other hand, are defined by the relative placement of precisely four α-carbons, so that they can be regarded as existing on the four-C^{α} length scale along the backbone. The same may be true of one of the two structural elements that together constitute a β-bulge.[2]

The observation of characteristic structures in folded proteins and the postulated folding mechanism together raise a basic question: What can be learned about protein folding by a systematic study of known protein structures on successive length scales? The goal of the work that forms the subject of this Account is to address this question.

The Differential-Geometric Representation

The primary tool for the study of protein structure on a given length scale is a representation or mathematical description that functions on that scale. This is, in principle, only a matter of convenience, since *any* representation of molecular structure contains information about structure on the length scale of interest. It should be emphasized, however, that the extraction of relevant information from an inappropriate representation can be a tedious procedure, which makes an intuitive picture of the data difficult to obtain. Thus, the distance-matrix representation, in which the structure of the molecule is specified by giving the distances between all pairs of atoms, is best suited to

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