DISTANCE MEASUREMENTS BETWEEN THE METAL-BINDING SITES IN THERMOLYSIN USING TERBIUM ION AS A FLUORESCENT PROBE

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SUMMARY: A single terbium ion has been introduced into thermolysin replacing two of the four calcium ions, and the fluorescence properties of the protein-bound terbium have been studied. The fluorescence of Tb^{+3} is tremendously enhanced ($\sim 7 \times 10^3$) upon binding and is significantly quenched when divalent cobalt is substituted for the zinc ion normally found in the enzyme. By use of the Förster equation for energy transfer the distance between the protein-bound Tb^{+3} and Co^{+2} in the active site was calculated to be 13.6±0.5 A. This agrees closely with the value of 13.9 A obtained from the crystal structure and suggests that energy transfer between the two metal ions bound to the protein takes place by a dipole-dipole mechanism.

In recent years lanthanide ions have been used as structural probes in various protein systems (1-3). They have been shown to substitute for the calcium ion, and their magnetic and spectroscopic properties aid in determining the environment about the metal ion (4-8). The lanthanide ions have also aided in the determination of the crystal structure of thermolysin (8,9).

Thermolysin, a proteolytic enzyme of MW 37,500, binds four calcium ions which stabilize the enzyme against denaturation and autolysis (10,11). One zinc ion is bound in the active site and is required for enzyme activity. Cobalt(II) can be substituted for the zinc ion with a subsequent doubling in the rate of hydrolysis of the synthetic substrate furylacryloylglycyl leucine amide (FAGLA) (12). It was shown earlier that in the thermolysin crystal a single lanthanide ion will replace the two calcium ions which are in close proximity (7). We have substituted a single Tb^{+3} for these two Ca^{+2} ions in thermolysin in solution. The fluorescence spectrum of protein-bound Tb^{+3} is

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partially quenched when Co^{+2} replaces Zn^{+2} due to energy transfer between the two protein-bound metal ions. From this decreased fluorescence intensity the distance between the protein-bound Tb^{+3} and Co^{+2} has been calculated.

Theory: Förster proposed that singlet-singlet energy transfer occurs by a resonance interaction of a dipole pair consisting of an energy donor and acceptor chromophore (13). Quantitatively, R_o, the distance (in A) at which energy transfer is 50 percent efficient, is related to spectroscopic and geometrical variables by the expression:

$$R_o = 9.79 \times 10^3 \, (Jn^{-4}K^2Q)^{1/6} \qquad (1)$$

in which J is the spectral overlap between the fluorescent donor, Tb^{+3}, and the acceptor, Co^{+2}, n is the refractive index of the medium, K is the orientation factor of the dipole pair and Q is the quantum yield of the donor in the absence of energy transfer (14). The singlet-singlet energy transfer (T) for a donor-acceptor pair at a defined distance, R, is given by:

$$\frac{F_{Co^{+2}}}{F_{Zn^{+2}}} = 1-T = \frac{1}{1+\left(\frac{R_o}{R}\right)^6} \qquad (2)$$

where $F_{Zn^{+2}}$ is the terbium fluorescence intensity of Zn-thermolysin and $F_{Co^{+2}}$ is the terbium fluorescence intensity of thermolysin whose zinc has been replaced by cobalt. R_o can be calculated from eq (1), $F_{Co^{+2}}/F_{Zn^{+2}}$ is measured experimentally and R, the distance between the terbium and cobalt ions, can be calculated from eq (2).

Experimental: Three times recrystallized thermolysin (Sigma) was purified using the method of Vallee (15). Terbium chloride was prepared by dissolving an excess of the oxide in Baker Ultra-pure HCl, filtering and standardizing with EDTA. Enzyme assays were done in 0.01 M HEPES, pH 7.5, 0.01 M $CaCl_2$, 1.0 M NaCl at 345 nm using FAGLA as substrate. All solutions used in the fluorescence measurements contained 0.01 HEPES buffer, 0.01 M $CaCl_2$, and 1.0 M NaCl at pH 7.5. Visible absorption spectral measurements were taken with 5 cm cells using the Cary 14 and the 0-0.1 A slidewire. Fluorescence measurements were obtained on an Aminco-Bowman Spectrophotofluorimeter with the ratio attachment. Cobalt and calcium ion concentrations were determined using the Varian AA-5 Atomic Absorption instrument. Lanthanide ion concentrations were determined by microtitration at pH 6.1 (0.2 M sodium acetate) using EDTA as a titrant with xylenol orange as the indicator. Thermolysin concentrations were determined from the absorbance at 280 nm (ε = 52,400 $M^{-1}cm^{-1}$).

Zinc-free thermolysin was prepared by dialyzing the enzyme against three

changes of 0.01 HEPES, pH 7.5, 0.01 M $CaCl_2$, 1.0 M NaCl and 2 x 10^{-3} M o-phenanthroline followed by dialysis (three changes) against the same solution without o-phenanthroline. The mono-terbium substituted enzyme was prepared by adding a 15-fold excess of Tb^{+3} to either apo- or Zn-thermolysin and allowed to equilibrate for 12 hours. Alternatively dialysis for 12 hours of thermolysin against 10^{-4} M Tb^{+3} and 10^{-2} M Ca^{+2} yielded an enzyme preparation containing two calcium ions and one terbium ion. The cobalt enzyme was prepared by adding a stoichiometric amount of Co^{+2} to the apo-enzyme. That the enzyme was saturated with cobalt was verified by activity measurements (12).

Results and Discussion: Metal ion analysis showed that the Tb-Zn-thermolysin had the following metal ion to enzyme mole ratios: 2.0 Ca^{+2} 1.0 Zn^{+3}, 1.0 Tb^{+3}; the cobalt substituted preparation showed the following analysis 2.0 Ca^{+2}, 1.0 Tb^{+3}, 0.008 Zn^{+2} and 1.0 Co^{+2}. These data agree well with Matthews and Weaver's (7) data showing the replacement of two Ca^{+2} by one lanthanide ion.

Figure 1 shows the fluorescence spectrum of Tb^{+3}-Zn^{+2}-thermolysin (upper) as compared to Tb^{+3}-Co^{+2}-thermolysin (lower) under the same conditions. It is apparent that the terbium ion fluorescence is partially quenched in the presence of Co^{+2}. The cobalt-thermolysin absorption spectrum (shown in Figure 2) had a maximum at 555 nm with a molar extinction coefficient of 55 $M^{-1}cm^{-1}$. Since the major Tb^{+3} fluorescence peak (λ_{max} = 545 nm) occurs within the envelope of the cobalt-thermolysin absorption spectrum, the overlap integral was calculated over the wavelength region of the major Tb^{+3} emission (532-562 nm). The value of J

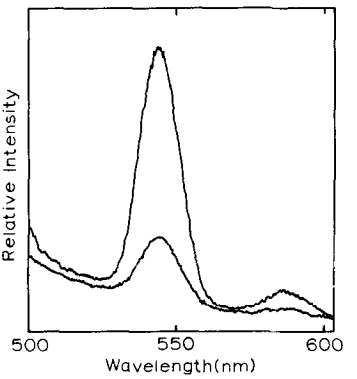

Figure 1 Tb^{+3} fluorescence spectrum of Zn^{+2}-thermolysin (top) and Co^{+2}-thermolysin (bottom). Thermolysin concentration was 4.77 x 10^{-6} M.

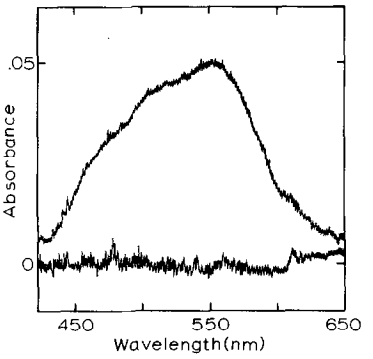

Figure 2 Co-Thermolysin visible absorption spectrum. Thermolysin concentration was 1.81 x 10^{-4} M in .01 M HEPES with .01 M $CaCl_2$, 1 M NaCl and pH 7.5.

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thus calculated was 4.07 x 10^{-16} cm^3M^{-1}. The refractive index, n, was taken to be 1.33. Since the Co^{+2} absorption band in the region of overlap with the terbium emission results from slightly split components of a triply degenerate transition corresponding effectively to an isotropically distributed acceptor moment, K^2 can be set equal to 2/3 (16,17). The only remaining quantity needed to solve eq. (1) for R_o is Q, the quantum yield of Tb^{+3} fluorescence. The Tb^{+3} fluorescence is measured by exciting at 280 nm to take advantage of the energy transfer from one or more tryptophans to the Tb^{3+} ion. Since the protein absorbs strongly at that wavelength, an estimate was made of the quantum yield of the protein-Tb^{+3} complex based on the quantum yield of model Tb^{+3} complexes. Table 1 shows quantum yields obtained for several different Tb^{+3} complexes. The values range from 0.152 to 0.401 and we have taken the average of 0.25 to use in our initial calculations. R_o was then calculated to be 16.3 A. $F_{Co^{+2}}/F_{Zn^{+2}}$ was found to be 0.253 from data in Figure 1. Solving Eq. (2), R, the distance between the two metal ions was found to be 13.6 A. Our calculated distance is then in close agreement with the value of 13.9 A reported from the crystal structure work.

The largest uncertainty in the above calculation is the quantum yield

TABLE 1

Quantum Yields of some Tb^{+3}-Chelates†

Chelate	Q	Chelate	Q
aspartate	0.349	citrate	0.152
glutamate	0.210	EDTA	0.401
acetate	0.172	DETPA	0.262

†Quantum yields were measured in 0.015 M HEPES, 0.1 M NaCl, pH 7.3 using eosin (Q = 0.19) as the standard (24). Excitation wavelength was 282 nm. A planimeter was used to measure the relative areas under the principal emission peak of Tb^{+3} (λ_{max} = 545 nm) and eosin (λ_{max} = 538 nm). Tb^{+3} concentration was 0.10 M. Aspartate, glutamate, citrate and acetate were 1.0 M, while ethylenediaminetetraacetic acid (EDTA) was 0.2 M and diethylenetetraaminepentaacetic acid (DETPA) was 0.25 M. Temperature was held constant at 25±.1°C.

estimate for the protein-Tb^{+3} complex being 0.25. However the environment of the lanthanide in the protein is not unlike the environment of the metal ion in these typical model complexes chosen, i.e., carboxyl complexes with variations in the number of water molecules surrounding the lanthanide ion (9). It is to be expected that the quantum yields calculated for these complexes should span the value for a Tb^{3+} ion in most metalloprotein complexes. Any error introduced due to the value of Q chosen should be rather small since R_o is dependent on the sixth root of Q, and thus R_o is relatively insensitive to rather large changes in the quantum yield. Distance calculations for R using the high and low vlues (0.152 and 0.401) for Q listed in Table 1 are 12.5 A and 14.7 A respectively, both of which are still in reasonable agreement with the x-ray work. The value of 0.25 for Q which we used is also in close agreement with the value 0.24 determined for Tb^{+3} bound to transferrin (18).

The fact that the distance we have calculated between the two metal ions is in such close agreement with the x-ray crystallographers measurements (9,19) is consistent with the transfer of energy from protein-bound Tb^{+3} to Co^{+2}

occurring by a dipole-dipole mechanism. This is particularly important since Tb^{+3} ion is being increasingly used as a biological fluorescent probe and in view of the fact that there has been controversy as to whether transfer of energy between lanthanide ions occurs by a dipole-dipole (R^6 dependence), a dipole-quadrupole (R^8 dependence) or a quadrupole-quadrupole (R^{10} dependence) mechanism (20-23). To our knowledge this is the first demonstration that energy transfer from a lanthanide ion to another chromophore is consistent with a dipole-dipole mechanism.

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REFERENCES

1. Darnall, D. W. and Birnbaum, E. R. (1970) J. Biol. Chem. 245, 6484-86.
2. Jones, R. and Dwek, R. A. (1974) Eur. J. Biochem. 47, 271-283.
3. Furie, B., Griffin, J. H., Feldman, R. J., Sokoloski, E. A. and Schechter, A. N. (1974) Proc. Nat. Acad. Sci. U.S. 71, 2833-2837.
4. Darnall, D. W. and Birnbaum, E. R. (1973) Biochemistry 12, 3489-3491.
5. Gomez, J. E., Birnbaum, E. R. and Darnall, D. W. (1974) Biochemistry 13, 3745-3750.
6. Abbott, F., Darnall, D. W. and Birnbaum, E. R. (1975) Biochem. Biophys. Res. Commun. 65, 241-247.
7. Matthews, B. W. and Weaver, L. H. (1974) Biochemistry 13, 1719-1725.
8. Colman, P. M., Weaver, L. H., Matthews, B. W. (1972) Biochem. Biophys. Res. Commun. 46, 1999-2005.
9. Matthews, B. W., Weaver, L. H., Kester,, W. R. (1974) J. Biol. Chem. 249, 8030-8044.
10. Feder, J., Garrett, L. R. and Wildi, B. S. (1971) Biochemistry 10, 4552-4555.
11. Drucker, H. and Borchers, S. L. (1971) Arch. Biochem. & Biophys. 147, 242-248.
12. Holmquist, B. and Vallee, B. L. (1974) J. Biol. Chem. 249, 4601-4607.
13. Forster, T. in Modern Quantum Chemistry, ed. O. Sinanoglu (1966) Vol. 3, p. 93, Academic Press, N. Y.
14. Cantor, C. H., Pechukas, P. (1971) Proc. Nat. Acad. Sci. U. S. 68, 2099-2101.
15. Latt, S. A., Holmquist, B. and Vallee, B. L. (1969) Biochem. Biophys. Res. Commun. 37, 333-339.
16. Latt, S. A., Auld, D. S. and Vallee, B. L. (1970) Proc. Nat. Acad. Sci. U. S. 67, 1383-1389.
17. Dale, R. E. and Eisinger, J. B. (1974) Biopolymers 13, 1573-1605.
18. Luk, C. K. (1971) Biochemistry 10, 2838-2843.
19. Matthews, B. W., Colman, P. M., Jansonius, J. N., Titani, K., Walsh, K. A. and Neurath, H. (1972) Nature N. B. 238, 41-43.
20. Kleinerman, M. (1969) J. Chem. Phys. 51, 2370-2381.
21. Dexter, D. L. (1953) J. Chem. Phys. 21, 836-850.
22. Nakazawa, E. and Shionoya, S. (1967) J. Chem. Phys. 47, 3211-3219.
23. Reisfeld, R. (1973) in Structure & Bonding Vol. 13 p. 53-98, Springer-Verlag, N. Y.
24. Weber, G. and Teale, F. W. (1957) Trans. Faraday Soc. 53, 640-648.