Identification of Metal-binding Sites in Rat Brain Calcium-binding Protein*

(Received for publication, August 18, 1995, and in revised form, October 18, 1995)

Timothy D. Veenstra, Myron D. Gross, Willi Hunziker, and Rajiv Kumar

From the Division of Epidemiology, School of Public Health, University of Minnesota, Minneapolis, Minnesota 55455, and the Department of Exploratory Research, Division of Vitamins and Fine Chemicals, F. Hoffman-LaRoche & Co., Ltd., CH-4002 Basel, Switzerland

Rat brain calbindin D28K binds 3 mol of terbium per mol of protein. To determine which of six EF-hand structures in the protein are responsible for terbium binding, we constructed three mutant forms of this protein, one lacking EF-hand 2 (RCaBP Δ2), the other lacking EF-hands 2 and 6 (RCaBP Δ2,6), and the third containing only EF-hands 3 and 4 (RCaBP Δ1,2,5,6), and examined their binding properties by fluorescence techniques. Full-length calbindin D28K and RCaBP Δ2 and RCaBP Δ2,6 bound 3 mol of terbium per mol of protein with high affinity. Thus, EF-hand domains 2 and 6 are not essential for calcium binding to the proteins, and an absence of EF-hands 2 and/or 6 does not alter the pattern of terbium binding to the protein. Using resonance energy transfer from tryptophan residues, one of the high affinity terbium-binding sites (site A) had a greater affinity than the other two sites (sites B and C) of each protein. Site A was filled before the other two sites. Calcium competition experiments showed that a greater amount of calcium was required to displace terbium from site A than from sites B or C. Energy transfer experiments from terbium to holmium showed that two of the terbium-binding sites are in close proximity while the third site is distant from the other two sites. To determine whether EF-hand 3 or 4 was responsible for binding of terbium, we examined the terbium-binding properties of a Δ1,2,5,6 RCaBP construct. The truncated protein RCaBP Δ1,2,5,6 contained a single terbium-binding site. Analysis of the terbium binding to RCaBP Δ1,2,5,6 construct showed that site 4 bound terbium, whereas site 3 did not. Analysis of the terbium-binding characteristics of the proteins suggests that EF-hands 1, 4, and 5 of rat brain calbindin D28K are responsible for terbium binding.

Calbindins are a group of vitamin D-dependent calcium-binding proteins that belong to the troponin-C family of proteins (1). This group of proteins occurs in several calcium-transporting tissues such as the intestine, kidney, and placenta as well as other tissues such as the brain, pancreas, bone, and parathyroid gland (2, 3). In some tissues, such as brain, these proteins are not vitamin D-dependent in adult animals. These proteins, like other members of the troponin-C family, bind calcium in “EF-hand” domains (4–7) which are composed of a helix-calcium-binding loop-helix motif. When calcium binds, conformational changes arise within the protein that can lead to the transmission of a biologically important signal (4, 5). Troponin-C, myosin light chain, and calmodulin alter enzyme or muscle activity by acting as secondary messengers (5, 8), while parvalbumin and intestinal calcium-binding protein probably function in calcium transport (9, 10). The plasma membrane calcium pump of intestinal cells is directly regulated by vitamin D3; the administration of vitamin D3 or 1,25-dihydroxyvitamin D3 directly increases plasma membrane calcium pump protein and messenger RNA in enterocytes (11). The plasma membrane calcium pump also contains a putative calcium-binding protein-binding site, and the addition of calbindins D to plasma membrane calcium pump preparations may directly alter calcium pump activity (12). These findings suggest that calbindins D, which are regulated by vitamin D in most tissues, could alter calcium transport by influencing plasma membrane pump activity.

Terbium can serve as an isomorphous replacement probe for calcium without causing significant structural changes in calcium-binding proteins (13). Since terbium possesses a high affinity for calcium-binding sites, calcium-binding proteins can bind terbium in a site-specific manner in stoichiometric amounts (13). When excited by light at an appropriate wavelength, protein-bound, but not aqueous, terbium is luminescent (14). Terbium emission intensity is the sum of the amount of protein-bound terbium times its quantum yield at each binding site (14). The quantum yield of terbium is a function of the number of coordinating groups with terbium and/or the affinity of terbium at a particular binding site (14). Terbium luminescence is quenchable by energy transfer to an appropriate donor atom such as holmium (14). This phenomenon has allowed the measurement of metal-metal distances in enzymes and is applicable to the determination of the spatial relationship between calcium-binding sites within proteins (14–17).

Rat brain calbindin D28K protein (RCaBP)

The abbreviations used are: RCaBP, rat brain calbindin D28K protein; PIPES, 1,4-piperazinediethanesulfonic acid.
we constructed three mutant proteins, one lacking EF-hand 2 (RCaBP Δ2), the other lacking EF-hands 2 and 6 (RCaBP Δ2,6), and one comprising only EF-hands 3 and 4 (RCaBP Δ1,2,5,6). Our studies have determined the calcium and lanthanide binding characteristics of RCaBP, RCaBP Δ2, RCaBP Δ2,6, and RCaBP Δ1,2,5,6 (residues 79 to 193 of the native protein) using lanthanide fluorescence techniques. These studies entailed measuring the fluorescence emission spectrum of terbium atoms bound to the proteins. The results show that recombinant full-length RCaBP, as well as both of the deletion mutant proteins, possess three high affinity terbium-binding sites. One of the high affinity terbium-binding sites is located in close proximity to a tryptophan residue. This terbium-binding site possesses a greater terbium affinity than the other sites based on terbium displacement with calcium. The truncated mutant RCaBP Δ1,2,5,6 contained a single high affinity terbium-binding site that was not located near a tryptophan residue.

**MATERIALS AND METHODS**

Deletion mutagenesis, expression, and purification of rat brain calcium-binding proteins were as described previously (8). Briefly, DNA sequences of the RCaBP full-length, Δ2, Δ2,6, and Δ1,2,5,6 plasmids were amplified by polymerase chain reaction techniques (19, 20) with Taq polymerase and polymerase chain reaction buffer (50 mM KCl, 10 mM Tris-HCl, 1.25 mM MgCl2, pH 8.3). The products were subcloned into the PCR vector using the Invitrogen TA Cloning Kit (Ref. 21; Invitrogen). Sequences encoding the RCaBPs were removed from the pCR vector using NdeI and BamHI restriction endonucleases and were ligated into gel-purified pET3a plasmid vector (Ref. 22; obtained from Dr. E. Wieben, Mayo Clinic, Rochester, MN) that had been cut with the same enzymes. The chimeric plasmids were transformed into competent Escherichia coli BL21(DE3) pLysS cells (Ref. 22; from Dr. E. Wieben). Protein expression was initiated by adding isopropyl-1-thio-β-D-galactoside to a final concentration of 0.4 mM. Protein production was assayed by SDS-polyacrylamide gel electrophoresis (23). The highest available grades of terbium(III) chloride (TbCl3) hexahydrate, holmium chloride (HoCl3) hexahydrate, and lanthanum chloride (LaCl3) heptahydrate were obtained from Aldrich and used without further purification. EDTA, CaCl2, and sesquioxalate PIPES were obtained from Sigma. The concentrations of the lanthanide solutions were determined by EDTA titrations in the presence of a xylene orange indicator (24). Protein concentration was determined by UV absorbance (E280 nm = 7.93 (18)) for full-length RCaBP and by amino acid analysis for RCaBP Δ2 (E280 nm = 8.17) and RCaBP Δ2,6 (E280 nm = 8.78). The concentration of RCaBP Δ1,2,5,6 was determined by dry weight and by protein assay using the Bradford method (25) (E1% 280 nm = 8.56). Purified expressed proteins were assessed by high performance liquid chromatography, amino acid analysis (26), and automated Edman degradation sequence analysis (15).

Fluorescence measurements were recorded on a Fluoromax fluorescence spectrometer (Spectrux Instruments, Edison, NJ). The geometry of the fluorescence detection was 90°. All measurements were recorded using a fused quartz cell with a 1.0-cm path length, and the sample temperature was maintained at 25 °C using a circulating water bath. Terbium was excited either directly (λem = 488 nm) or indirectly by energy transfer from a tryptophan residue (λexc = 290 nm). Energy transfer from tryptophan with light of 290 nm was used where terbium was excited indirectly to optimize signal intensity at 280 nm, while shifting second order light (2 λem + λexc) away from the emission of terbium (545 nm). Light with a wavelength of 290 nm also specifically excites tryptophan residues. Alternately, terbium was excited directly by 488 nm light. The emission intensity of terbium was recorded between 530 and 560 nm, with the maximum intensity at 544 nm. Peak height was used as a measure of peak area, and the intensity at 530 nm was plotted as the baseline. The excitation and emission bandwidths were 8 nm for all spectra in which terbium was excited directly and 4 nm for spectra in which terbium was excited by energy transfer from tryptophan.

In experiments where terbium was excited directly or by energy transfer from tryptophan residues, calcium and/or lanthanides were added to the protein in microliter amounts not exceeding 300 μl to a starting volume of 4 ml. In all experiments, solutions were allowed to equilibrate after additions of lanthanide or Ca2+ before measurements were taken. All experiments were done in 50 mM PIPES buffer (pH 6.7).

**RESULTS**

The amino acid sequence of full-length RCaBP is shown in Fig. 1. In the formation of the Δ2 mutant, residues from the end of EF-hand domain 1 to the end of the loop in EF-hand domain 2 were removed. This deletion brings EF-hands 1 and 3 in close proximity, separated only by a short linker segment. In the Δ2,6 mutant, the aforementioned residues were deleted along with residues from the end of EF-hand domain 5 to the end of the loop of EF-hand domain 6. The sequential relationship between EF-hands 3, 4, and 5 are not affected by the formation of either mutant protein Δ2 or Δ2,6.

The results of the titration of full-length RCaBP with terbium emission recorded by direct excitation are shown in Fig. 2. An increase in fluorescence emission intensity was observed with increasing amounts of terbium up to a [TbCl3]/[RCaBP] ratio of 3. This experiment was repeated with a higher concentration of terbium (14.0 μM) and the results were similar. The results of the terbium titration RCaBP Δ2 (4.23 μM)/RCaBP Δ2,6 (7.88 μM) are also shown in Fig. 2. In both of these mutant proteins, an increase in emission intensity was also observed up to a [TbCl3]/[protein] ratio of 3. In the native protein, the emissions profile suggests the presence of a single high affinity site and two lower affinity sites. In the case of RCaBP Δ2 and RCaBP Δ2,6, there appears to be two high affinity terbium-binding sites and a single lower affinity binding site. Neither of the high affinity terbium-binding sites in the mutant proteins appear to have an affinity as great as the high affinity site in the native protein. Overall, the results suggest that all three of these mutant proteins contain 3 terbium-binding sites, which do not possess equal affinities for terbium. These terbium-binding sites were arbitrarily designated A, B, and C.

Experiments were performed to determine the relative proximity of the terbium-binding sites to tryptophan (Trp) residues. Terbium can be excited by Förster dipole-dipole energy transfer from Trp or tyrosine (Tyr) residues (27, 28). Rat brain CaBP contains two tryptophan residues at positions 20 and 107, both of which are located within putative calcium binding domains (8). Tryptophan 20 is located within EF-hand 1, and Trp 107 is located in EF-hand 3.

The results of the Förster energy transfer measurements of RCaBP, RCaBP Δ2, and RCaBP Δ2,6 titrated with terbium are shown in Fig. 3. The three proteins show a sharp increase in terbium emission intensity up to a [TbCl3]/[protein] ratio of 1. Between a [TbCl3]/[protein] ratio of 1.0 and 1.5, a small increase in terbium emission intensity was observed in the titration of all three proteins with terbium. The sharp increase in terbium emission, up to a ratio of 1.0, suggests the existence of a terbium-binding site nearer a Trp residue with a greater terbium affinity than the other two sites.

The relative proximity of terbium-binding sites can be determined from quenching experiments with lanthanides. Protein-bound holmium atoms, close to protein-bound terbium, can quench terbium emission. Lanthanum is useful as a control to...
distinguish fluorescent quenching from displacement of terbium from metal-binding sites on the protein, because lanthanum does not quench terbium emission. The titration of protein-terbium complexes was monitored by direct excitation of terbium, which monitors all three sites, and by energy transfer from Trp which monitors site A. As seen in Fig. 4, the decrease in terbium fluorescence when the terbium-protein complexes were excited at 290 nm, was similar in the HoCl₃ and LaCl₃ titrations of all three proteins, suggesting no direct quenching from tryptophan in the presence of lanthanum or holmium. RCaBP-terbium complexes were titrated with HoCl₃ ([RCaBP] = 7.33 μM) (Fig. 4B) and LaCl₃ ([RCaBP] = 1.93 μM, [TbCl₃] = 1.79 μM, LnCl₃, lanthanide chloride).

The results of the titration of RCaBP Δ1,2,5,6 (7.33 μM) with terbium emission recorded by direct excitation are shown in Fig. 6. A sharp, steady increase in fluorescence emission intensity was observed with increasing amounts of terbium up to a [TbCl₃]/[RCaBP] ratio of 1. There was also a slight increase in fluorescence emission intensity at terbium to protein ratios greater than 1 is likely a result of nonspecific terbium binding. The results of this experiment suggest the presence of one terbium-binding site within RCaBP Δ1,2,5,6.

To determine the position of the high affinity terbium-binding site within RCaBP Δ1,2,5,6, the protein was titrated with terbium again, and the fluorescence emission was measured by energy transfer from tryptophan. There is a single tryptophan residue located in EF-hand 3 of this protein. The results of the Förster energy transfer measurements of RCaBP Δ1,2,5,6 titrated with terbium are shown in Fig. 7 compared with the results of the same experiment using full-length RCaBP. The concentrations of the proteins were identical, and all of the experimental conditions were the same in order to get an accurate comparison of the terbium emission intensities for both proteins. There is a weak gradual increase in the terbium emission intensity at lanthanide to protein ratios between 1 and 2. The increase in terbium emission intensity at terbium to protein ratios greater than 1 is likely a result of nonspecific terbium binding. The results of this experiment suggest the presence of one terbium-binding site within RCaBP Δ1,2,5,6.

The relative affinities of the terbium-binding sites were measured by determining the amount of calcium required to decrease the terbium fluorescence intensity by 50%. The results of the titration of terbium-protein complexes with calcium...
are shown in Figs. 8 and 9. In Fig. 8, the terbium emission is measured by energy transfer from tryptophan. In the case of all three proteins, it required a calcium chloride (CaCl$_2$) to terbium ratio of at least 200 to result in a 50% decrease in terbium fluorescence intensity. When the same experiment was repeated with terbium emission measured by direct excitation (Fig. 9), it required only a 10-fold excess of CaCl$_2$ to terbium to cause a 50% decrease in terbium emission. This result suggests that site A has a greater terbium affinity than the other two sites. Because calcium displaces terbium from metal-binding sites, it can be concluded that these sites are also calcium-binding sites.

**DISCUSSION**

This study provides direct evidence characterizing the number and position of the terbium- and calcium-binding sites on rat brain CaBP. Through the use of deletion mutagenesis, it is possible to eliminate specific putative binding sites within the protein. By comparing the terbium and calcium binding characteristics of the mutant proteins to the native protein, the position of the functional EF-hand domains can be determined.

The results of our fluorescence studies suggest that RCaBP, as well as RCaBP$^\Delta 2$ and RCaBP$^\Delta 2,6$, binds 3 mol of terbium/mol of protein; each contains three terbium-binding sites possessing different terbium affinities. The three terbium-binding sites have been arbitrarily designated A, B, and C (18). Full-length RCaBP contains six putative EF-hand domains based on analysis of its primary sequence. These EF-hands can be designated 1 through 6 based on their expected order of appearance from the amino terminus of the protein. Since the two mutant forms of the protein lack EF-hand 2 or both 2 and 6 and bind the same number of equivalents of terbium as the native protein, EF-hands 2 and 6 are not essential for terbium binding within RCaBP. Therefore, the possible terbium-binding sites within RCaBP are EF-hands 1, 3, 4, and 5.
form an binding sites, EF-hands 1 and 3, offers some explanation why binding domains within RCaBP. Considered that EF-hands 1, 4, and 5 are both terbium and calcium binding sites are evident. Therefore, it can be concluded that a single terbium-binding site is observed. When terbium is excited indirectly, no energy transfer occurs over short distances relative to the size of proteins (14). Förster energy transfer between Trp and terbium can be used to distinguish between terbium-binding sites since the energy transfer occurs over short distances relative to the size of proteins (14). The terbium emission profile shows that the terbium-binding site in close proximity to a Trp residue is filled before the other terbium-binding sites and therefore possesses a higher terbium affinity than the remaining two sites. Two Trp residues are located within putative EF-hand domains in RCaBP and mutant proteins. Tryptophan 20 is located within the first helical region of EF-hand 1, and Trp-107 is located within the first helical region of EF-hand 3. Both residues are located in similar positions within the helical regions, approximately equal distances from the center of the binding site. Therefore, either EF-hand 1 or 3, but not both sites, must bind terbium. Our results of the titration of RCaBP A1,2,5,6 with terbium strongly suggest that EF-hand 3 does not bind terbium. When RCaBP A1,2,5,6 is titrated with terbium and terbium emission is measured by direct excitation, the presence of a single terbium-binding site is observed. When terbium is excited indirectly, no terbium-binding sites are evident. Therefore, it can be concluded that EF-hands 1, 4, and 5 are both terbium and calcium binding domains within RCaBP.

Analysis of primary structures of the two potential terbium-binding sites, EF-hands 1 and 3, offers some explanation why EF-hand 3 does not bind terbium. The residues comprising the second helical region of EF-hand 3 do not have a propensity to form an α-helix (19). This suggests that EF-hand 3 does not possess the typical EF-hand structure and therefore is unable to bind terbium. Although one cannot absolutely conclude that the terbium binding characteristics of RCaBP A1,2,5,6 are identical with their counterparts in the native protein, it has been shown that a truncated form of RCaBP containing only EF-hands 1 and 2 binds 1 mol of terbium per mol of protein as it does in the native protein (16). This suggests that pairs of EF-hands excised from RCaBP can bind terbium independently.

Studies utilizing holmium to quench terbium fluorescence support the identification of EF-hands 1, 4, and 5 as the terbium-binding sites. The interpretation of the results of the titration of the terbium-protein complexes with holmium and lanthanum assumes there is no strong sites specificity for holmium versus lanthanum. Holmium quenched terbium emission when terbium was excited by Förster energy transfer, thereby monitoring terbium fluorescence of site A. This implies that terbium bound to site A is greater than 12 Å away from the remaining lanthanide-binding sites. Holmium was able to quench terbium emission when terbium was excited directly, monitoring the terbium fluorescence of all three sites. The holmium quenching behavior was similar in all three proteins in which quenching studies were done. Although the three-dimensional structure of RCaBP is not known, based on the location of the proposed EF-hand domains, it is more likely that domains 4 and 5 are in closer proximity than either of these domains and EF-hand 1.

The identification of three metal-binding sites essentially agrees with the number of high affinity calcium-binding sites originally found for chick intestinal CaBP (7) and reported later in fluorescence studies of the protein (28). In this and other previous studies (31), the presence of a fourth metal-binding site was suggested. In the fluorescence studies, the presence of high and low affinity terbium-binding sites similar to those found in RCaBP are reported. In neither of these studies was the precise location of the terbium-binding sites conclusively determined. The results presented here not only determine the number of metal-binding sites within the protein but also indicate the locations of these binding sites.

The formation of the mutant proteins results in a significant replacement and rearrangement of the helices making up the EF-hand domains 1 and 5 in the native protein (Fig. 1). The results of our experiments suggest that this has little effect on the calcium-affinity of the calcium-binding sites. Studies measuring the calcium affinity of site III of troponin C have shown that very minor amino acid substitutions in the helical region of this EF-hand domain have a significant effect on the calcium dissociation constant (32). Therefore, it is unlikely that the complete rearrangement of the helices seen in the mutant proteins has absolutely no effect on the calcium affinity of the EF-hand domains. The deletion of EF-hand 2 should result in a decrease in the lanthanide affinity of EF-hand 1 unless the rearrangement of the EF-hand domains compensates for the deletion of domain 2, such that EF-hand 3 can substitute for EF-hand 2 and preserve the conformation of the metal-binding site. Similarly, in the Δ2,6 mutant, EF-hand 3 and EF-hand 5 might substitute for EF-hands 2 and 6. In the native protein, it would be expected that EF-hand domains 1 and 2, 3 and 4, and 5 and 6 form three pairs of EF-hands. In the mutated proteins, a reshuffling of the domains may have occurred such that EF-hands 1 and 3 and 4 and 5 comprise the EF-hand pairs. The observation that the lanthanide affinity of EF-hand 1 is not perturbed by the deletion of EF-hand 2 does not detract from the observation that EF-hand pairing is necessary for metal binding. More detailed experiments must be performed in order to clarify any changes in calcium affinity as a result of the mutation of the native RCaBP protein.

The conclusion reached in this study is that EF-hands 1, 4, and 5 of RCaBP have been identified as terbium and calcium binding domains. EF-hand 1 has a greater terbium affinity than EF-hands 4 or 5 and is separated from this pair of EF-hands by at least 12 Å. Studies are presently underway attempting to determine the effects of calcium binding on the conformation of the protein and also to identify any protein-protein interactions involving RCaBP.
REFERENCES

1. Barker, W. C., Ketcham, L. K., and Dayhoff, M. O. (1977) in Calcium Binding Proteins and Calcium Function (Wasserman, R. H., Corradino, R. A., Carafoli, E., Krebsinger, R. H., MacLennan, D. H., and Siegel, F. L., eds) pp. 73–75, Elsevier Science Publishing Co., New York

2. Feher, J. J. (1983) Am. J. Physiol. 244, F303–F309

3. Gross, M. D., and Kumar, R. (1990) Am. J. Physiol. 259, F195–F209

4. Babu, S. Y., Sock, J. S., Greenough, T. J., Bugg, C. E., Means, A. R., and Cook, W. J. (1985) Nature 315, 37–40

5. Kretsinger, R. H. (1976) Annu. Rev. Biochem. 45, 239–266

6. Sundaralingam, M., Bergstrom, R., Strasburg, G., Rao, S. T., Roychowdury, P., Greaser, M., and Wang, B. C. (1985) Science 227, 945–948

7. Szefienyi, D. M. E., Obendorf, S. K., and Moffat, K. (1981) Nature 294, 327–332

8. Kumar, R., Hunziker, W., Gross, M., Naylor, S., Londowski, J. M., and Schaefer, J. (1994) Arch. Biochem. Biophys. 308, 311–317

9. Pechere, J. F., Derancourt, J., and Haiech, J. (1977) FEMS Lett. 111, 111–114

10. Wasserman, R. H. (1980) in Calcium Binding Proteins: Structure and Function (Siegel, F. L., Carafoli, E., Kretsinger, R. H., MacLennan, D. H., and Wasserman, R. H., eds) pp. 357–361, Elsevier Science Publishing Co., New York

11. Cai, Q., Chandler, J. S., Wasserman, R. H., Kumar, R., and Penniston, J. T. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 1345–1349

12. Reiter, P. D., Christakos, S., and Vanaman, T. C. (1992) FEMS Lett. 127, 127–131

13. Horrocks, W. DeW., J. r. (1982) Adv. Inorg. Biochem. 4, 201–261

14. Horrocks, W. DeW., J. r., and Sudnick, D. R. (1981) Acc. Chem. Res. 14, 383–392

15. Hewick, R. M., Hunkapillar, M. W., Hood, L. E., and Dreyer, W. J. (1981) J. Biol. Chem. 256, 7990–7997

16. Gross, M. D., Gonski, M., Tsarbaopoulos, A., and Hunziker, W. (1993) J. Biol. Chem. 268, 20917–20922

17. Rhee, M.-J., Sudnick, D. R., Arkle, V. K., and Horrocks, W. DeW., J. r. (1981) Biochemistry 20, 3328–3334

18. Gross, M. D., Nelsestuen, G. L., and Kumar, R. (1987) J. Biol. Chem. 262, 6539–6545

19. Gross, M. D., Kumar, R., and Hunziker, W. (1988) J. Biol. Chem. 263, 14426–14432

20. Innis, M. A., Geffand, D. H., Sninsky, J. J., and White, T. J. (1990) PCR Protocols: a Guide to Methods and Applications, pp. 3–12, Academic Press, San Diego, CA

21. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) Methods Enzymol. 185, 60–89

22. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1989) in Molecular Cloning: a Laboratory Manual, 2nd Ed., pp. 1.76–1.81, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

23. Lammli, U. K. (1970) Nature 227, 680–685

24. Birnbaum, E. R., and Sykes, B. D. (1978) Biochemistry 17, 4965–4971

25. Bradford, M. (1976) Anal. Biochem. 72, 248–254

26. Biddinger, B. A., Cohen, S. A., and Tarvia, T. L. (1984) J. Chromatogr. 336, 93–104

27. Förster, T. (1948) Ann. Phys. (Leyszig) 2, 55–75

28. Förster, T. (1965) in Modern Quantum Chemistry (Simenoglu, O., ed) Part III, pp. 93–137, Academic Press, New York

29. Horrocks, W. DeW., J. r., Holmquist, B., and Vallee, B. L. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 4764–4768

30. Horrocks, W. DeW., J. r., and Collier, W. E. (1981) J. Am. Chem. Soc. 102, 2856–2862

31. Bredderman, P., and Wasserman, R. H. (1974) Biochemistry 13, 1687–1694

32. Shaw, G. S., Hedges, R. S., and Sykes, B. D. (1991) Biochemistry 30, 8339–8347

33. Foerster, T. (1948) Ann. Phys. (Leyszig) 2, 55–75

34. Förster, T. (1965) in Modern Quantum Chemistry (Simenoglu, O., ed) Part III, pp. 93–137, Academic Press, New York

35. Horrocks, W. DeW., J. r., Holmquist, B., and Vallee, B. L. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 4764–4768

36. Horrocks, W. DeW., J. r., and Collier, W. E. (1981) J. Am. Chem. Soc. 102, 2856–2862

37. Bredderman, P. J., and Wasserman, R. H. (1974) Biochemistry 13, 1687–1694

38. Shaw, G. S., Hedges, R. S., and Sykes, B. D. (1991) Biochemistry 30, 8339–8347

39. Horrocks, W. DeW., J. r., Holmquist, B., and Vallee, B. L. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 4764–4768

40. Horrocks, W. DeW., J. r., and Collier, W. E. (1981) J. Am. Chem. Soc. 102, 2856–2862

41. Bredderman, P. J., and Wasserman, R. H. (1974) Biochemistry 13, 1687–1694

42. Shaw, G. S., Hedges, R. S., and Sykes, B. D. (1991) Biochemistry 30, 8339–8347
