discussions. Acknowledgment is made to the donors of the Petroleum Research Fund, administered by the American Chemical Society, for the partial support of this work. Additional support was provided by Davidson College.

Department of Chemistry
Davidson College
Davidson, North Carolina 28036

Ruth Freitag Beeston*
Steven L. Larson
Michael C. Fitzgerald

Received June 7, 1989

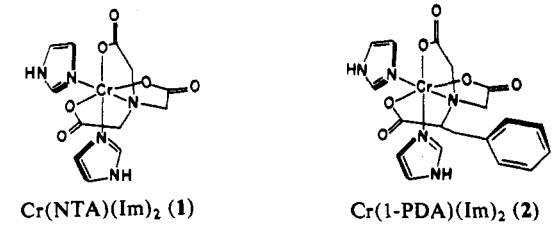
Protein Site Recognition by Chromium(III) Complexes Probed through NOE Quenching

We have recently become interested in the general principles behind the binding of inorganic substrates to protein molecules as a complement to ongoing work in this laboratory examining the interactions between transition-metal coordination complexes and DNA.[1] An elucidation of the principles of site recognition is the first step toward the rational design of both inorganic drugs as well as site-specific transition-metal labels and probes for proteins. Inorganic complexes may be particularly well suited to such investigations owing to their well-defined coordination geometries and the richness in spectroscopic probes that may be applied. Accordingly, we have synthesized a series of transition-metal-centered molecules containing both hydrogen-bonding moieties and pendant amino acid side chains. We have chosen to study their binding with hen egg white (HEW) lysozyme due to its well-characterized structure and complete NMR assignment.[2] We have discovered that selective paramagnetic quenching of nuclear Overhauser effects (NOE's) provides a sensitive means to map binding sites and to discriminate among binding interactions.

The quenching of NOE's between nuclei by paramagnets is described in both the experimental[3] and theoretical[4] literature. The coupling of unpaired electron spins to proton populations perturbed from spin equilibrium in the NOE experiment efficiently relaxes the system to equilibrium, circumventing the cross-relaxation pathways crucial to the observation of the NOE. As paramagnetic effects are distance dependent, only NOE's to protons in close proximity to the paramagnetic center are affected, allowing selective reduction of NOE signals to protons in the binding region. Hence, binding of a small complex containing a paramagnetic transition-metal center should cause diminution of the intensity of Overhauser effects to protons only in the binding site.

Surprisingly, NOE quenching has not been exploited as a binding site mapping technique on macromolecules.[5,6] We report here the use of this method to map specifically binding interactions between two related inorganic complexes containing pendant amino acid side chains and HEW lysozyme.

$Cr(NTA)(Im)_2$ (**1**) and $Cr(1\text{-}PDA)(Im)_2$ (**2**)[7] were synthesized and characterized by FABMS, IR spectroscopy, and elemental analyses, and the structure of **2** was determined by X-ray crystallography.[8] Chromium(III), which is one of the classic "shiftless" relaxation agents,[5b] is expected to have the same magnetic properties in both **1** and **2**, given that the primary coordination sphere is identical in each case. Under the conditions of these experiments, aquation of the complexes, with loss of imidazole, occurs.[9] Protein dialysis experiments indicate that direct coordination of the complex to the protein is unlikely.



$Cr(NTA)(Im)_2$ (**1**)

$Cr(1\text{-}PDA)(Im)_2$ (**2**)

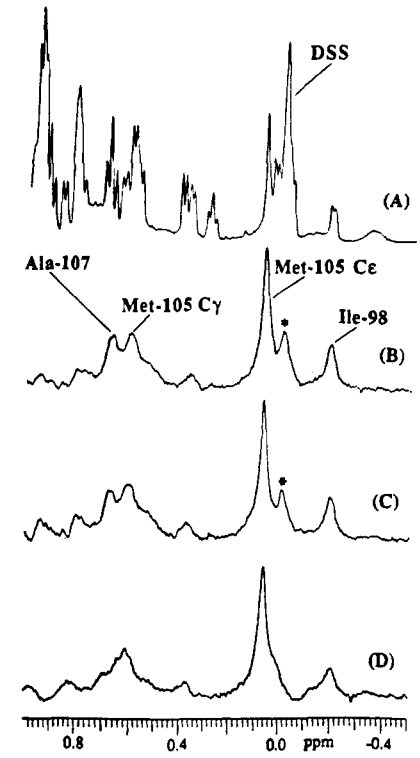


Figure 1. (A) ^1H resolution-enhanced 400-MHz NMR spectrum of HEW lysozyme for comparison to NOE spectra. (B) NOE difference spectrum of lysozyme obtained by irradiating Trp-105 C5H at 6.5 ppm in the absence of metal complex. The sign of the NOE's is negative. (C) NOE difference spectrum as in part B but in the presence of a 5:1 ratio of protein to complex **1**. (D) NOE difference spectrum as in part B but in the presence of a 5:1 ratio of protein to complex **2**. All samples were 3 mM in lysozyme in D_2O at pH 3.8, 35 °C. The starred peak is a subtraction artifact due to the DSS standard resonance. Line broadening of 2 Hz has been applied. Note that the NOE to Ala-107 is weakly quenched for **1** and dramatically quenched for **2**. NOE's to Ile-98 also show quenching while no quenching is evident of NOE's to Met-105.

Binding site mapping experiments were conducted following paramagnetic difference[10] (PD) spectroscopy, since it was necessary first to establish which lysozyme ^1H resonances were strongly affected by metal binding and which were unaffected.[11]

(1) Barton, J. K. *Science* **1986**, *233*, 727–734.
(2) Redfield, C.; Dobson, C. M. *Biochemistry* **1988**, *27*, 122–136.
(3) Freeman, R.; Pachler, K. G. R.; LaMar, G. N. *J. Chem. Phys.* **1972**, *55*, 4586–4593.
(4) Gutowsky, H. S.; Natusch, D. F. S. *J. Chem. Phys.* **1972**, *57*, 1203–1215.
(5) Chromium(III) complexes have previously been used for blanket quenching of all NOE's in quantitative ^{13}C NMR experiments. See, for example: (a) Levy, G. C.; Cargioli, J. D. *J. Magn. Reson.* **1973**, *10*, 231–234. (b) Levy, G. C.; Edlund, U.; Hexem, J. G. *J. Magn. Reson.* **1975**, *19*, 259–262. (c) Levy, G. C.; Komorosky, R. A. *J. Am. Chem. Soc.* **1974**, *96*, 678–681.
(6) A recent report utilized NOE quenching to study the binding of a paramagnetic ion to an organic molecule (Gaggelli, E.; Tiezzi, E.; Valensin, G. *J. Chem. Soc., Faraday Trans. 2* **1988**, *84*, 141–147).
(7) Abbreviations: NTA = nitrilotriacetate trianion; 1-PDA = 1-phenylalanine-*N,N*-diacetic acid trianion; Im = imidazole; DSS = 2,2-dimethyl-2-silapentane-5-sulfonate.
(8) FABMS (nitrobenzyl alcohol matrix): (**1**) M + H = 377; (**2**) M + H = 467. Anal. Calcd for (**1**) $Cr(NTA)(Im)_2{\cdot}3H_2O$: C, 33.49; H, 4.68; N, 16.28. Found: C, 33.55; H, 4.62; N, 16.28. Calcd for (**2**) $Cr(1\text{-}PDA)(Im)_2{\cdot}CH_3OH{\cdot}{}^1/_2C_7H_8$ (from crystal structure): C, 51.84; H, 5.18; N, 12.86. Found: C, 51.00; H, 5.32; N, 13.15. IR (KBr pellet): (**1**) ν_{COO}(asym) = 1661, 1639 cm^{-1}; (**2**) ν_{COO}(asym) = 1641 cm^{-1}. The synthesis, full characterization, crystal and molecular structure will be the subject of a future publication.
(9) Preliminary findings suggest that approximately one imidazole is lost per complex over the course of the experiment. The chromium–imidazole bond is known to be labile. See, for example: Winters, J. A.; Caruso, D.; Shepherd, R. E. *Inorg. Chem.* **1988**, *27*, 1086–1089.
(10) Campbell, I. D.; Dobson, C. M.; Williams; R. J. P.; Xavier, A. V. *J. Magn. Reson.* **1973**, *11*, 172–181.

0020-1669/89/1328-4189$01.50/0

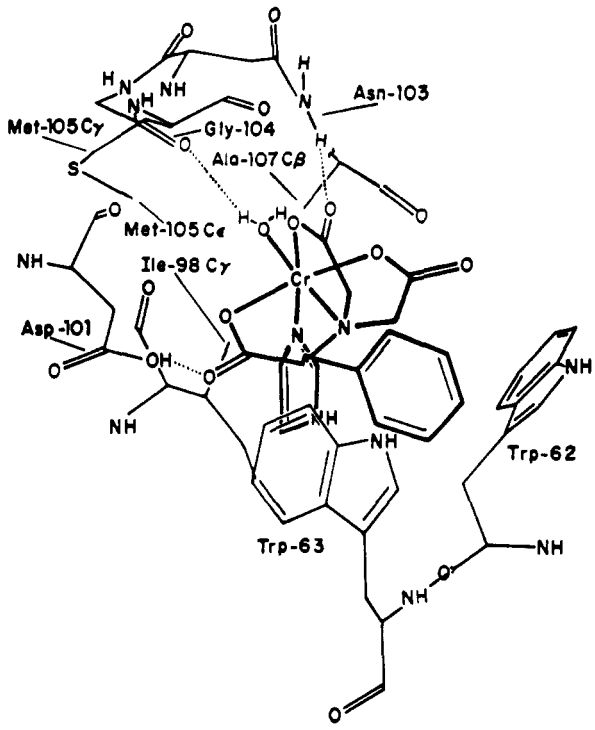


Figure 2. Binding model for **2** bound in the HEW lysozyme active cleft based upon NOE quenching and PD data. Coordinates for the protein are based upon its crystallographic determination. The proposed hydrogen bonds to the carboxylate of Asp-101, the amide group of Asn-103, and the peptide carbonyl of Gly-104, in addition to the aromatic–aromatic interaction between the pendant phenylalanine side chain of **2** and Trp-62 and/or Trp-63, orient the substrate molecule so that the Cr(III) center is in close proximity to the methyl group of Ala-107.

Then 1-D NOE's were measured[12] between protons observed to be unaffected by metal complex binding and protons of interest. Figure 1 illustrates NOE difference spectra[13] measured by irradiating Trp-108 C5H in the presence and absence of **1** and **2**. These spectra show preferential quenching of NOE's to both Ala-107 $C\beta H_3$ and Ile-98 $C\gamma_2H_3$; NOE's to Met-105 $C\gamma H_2$ and $C\epsilon H_3$ are unaffected. A concentration dependence study (Figure S1, supplementary material) on these NOE's shows that the affected NOE peaks are quenched as a function of increasing concentration (as expected) with no evidence of chemical shift changes. The NOE to Ala-107 $C\beta H_3$ is completely quenched (0% NOE enhancement) by **2** at a protein to quencher ratio of 2.5:1; at this ratio, some 10% of the original NOE enhancement remains in quenching with **1**.

Similar experiments using irradiation at Met-105 $C\beta H_2$, Leu-17 $C\delta_2H_3$, Ile-98 $C\gamma_1H_2$, Trp-28 C5H and Asn-59 $C\alpha H$, along with broadening effects observed in PD spectra,[14a] allow elucidation of a single binding site for both **1** and **2** within the active cleft of lysozyme. The most strongly perturbed protons are located on Ala-107, Trp-63, and Ile-98, with Ala-107 showing the largest effect overall. The binding region is delimited by Asn-59 and Trp-108. The $C\alpha H$ of Asn-59 shows no perturbation. Only a slight effect is observed at the $C\beta H$ protons of Asn-59, and no effect is seen at Trp-108 (C4H, C5H, or C6H).

Comparisons between quenching efficiencies of **1** and **2** illustrate how the effect of differing recognition elements on the probe complex may be observed via NOE quenching. As Figure 1 illustrates, both **1** and **2** quench active-site NOE's, in particular that of Trp-108 to Ala-107, yet **2** does so more efficiently. This observation, taken in conjunction with NOE quenching data to other protons spanning the binding site, indicates a higher binding site occupancy for **2** over **1**.[14b] The increased binding of **2** in this site may be attributed to an aromatic–aromatic interaction between the pendant phenylalanine ring of **2** and either Trp-62, Trp-63, or both. Aromatic–aromatic interactions have been noted between aromatic side chains in protein and peptide crystal structures and between aromatic rings on proteins and their bound substrates.[15,16] The orientation that this aromatic–aromatic interaction imposes on the complex with respect to the protein places the metal center closest to Ala-107, consistent with the strong effect observed there[17] (see Figure 2). The comparison of **1** to **2** illustrates how the addition of a recognition element to the set of interactions common to both molecules that govern binding at this site can be detected by NOE quenching.

We have demonstrated the use of the novel technique of NOE quenching by small inorganic complexes to characterize structurally their binding to HEW lysozyme. Comparison of the NOE quenching of structurally related probes allows examination of the effects of individual recognition elements on the molecules. As additional metal-centered targeting agents are designed, we anticipate the development of this technique into a sensitive tool for both the structural identification of small molecule binding sites and the elucidation of the factors involved in the recognition of protein sites by transition-metal complexes.

Acknowledgment. We are grateful to the National Science Foundation (Presidential Young Investigator Grant to J.K.B., No. CHE83-51017) for their financial support of this work. We also thank Dr. C. Turner for his expert assistance with the NMR experiments.

Registry No. **1**, 123266-17-1; **2**, 123266-18-2; lysozyme, 9001-63-2.

Supplementary Material Available: A figure showing NOE quenching as a function of concentration (1 page). Ordering information is given on any current masthead page.

(11) We also observe another binding site of comparable strength in the vicinity of His-15.

(12) Poulsen, F. M.; Hoch, J. C.; Dobson, C. M. *Biochemistry* **1986**, *19*, 2597–2601.

(13) A typical experiment was conducted as follows. Grade I lysozyme (Sigma) was exhaustively dialyzed against water at pH 3.0 and then lyophilized. A weighed amount of lyophilized protein was dissolved in D_2O and was then heated to 80 °C for 10 min to exchange fully all labile protons. After cooling, 2 mg of DSS were added as internal standard and the pH was adjusted with DCl/NaOD until the pH meter reading was 3.8. The protein concentration was determined by UV absorbance, using $\epsilon_{280} = 37\,600\ M^{-1}\ cm^{-1}$. Protein concentrations were 3 mM. Freshly prepared stock solutions of metal complex, made up by weight in CD_3OD, were added to protein to yield a final protein/complex ratio of 5:1 (<2% CD_3OD). NOE's were then measured at 400 MHz at 35 °C by using an irradiation time of 1 s and an acquisition time of 0.5 s. A total of 1200 transients were acquired per irradiation with interleaving (block size = 40).

(14) (a) Bocarsly, J. R.; Barton, J. K. Unpublished data. (b) While differential quenching of NOE's to any individual proton by **2** over **1** may be explained by either a higher binding constant or a closer positioning of **2** to the proton in question, examination of the effects on a distribution of protons about the putative binding site can differentiate between the two possibilities. If the difference in quenching is due to a change in average quencher position, then quenching of some protons in a roughly spherical distribution should *increase* with **2** versus **1**, while others should *decrease*; the chromium complex in moving toward some protons must move away from others. If, however, the difference between **2** versus **1** is due to a change in binding constant, then all protons affected should be quenched more by **2** than by **1**. The latter possibility is experimentally observed. Quenching at Ala-107 $C\beta H_3$, Ile-98 $C\beta H$, Ile-98 $C\gamma_2H_3$, Trp-63 C2H, Trp-62 C2H, Asn-59 $C\beta_1H$ and Asn-59 $C\beta_2H$ is greater in all cases for **2** versus **1**. We therefore conclude that **2** has a higher binding constant than **1** at this site.

(15) (a) Burley, S. K.; Petsko, G. A. *Science* **1985**, *229*, 23–28. (b) Burley, S. K.; Petsko, G. A. *J. Am. Chem. Soc.* **1986**, *108*, 7995–8001.

(16) (a) Ringe, D.; Seaton, B. A.; Gelb, M. H.; Abeles, R. H. *Biochemistry* **1985**, *24*, 64–68. (b) Lipscomb, W. N. *Proc. Robert A. Welch Found. Conf. Chem. Res.* **1971**, *15*, 140.

(17) We note that a variety of other nonclassical aromatic interactions have been discussed and may be important in this case. See, for example: Burley, S. K.; Petsko, G. A. *FEBS Lett.* **1986**, *203*, 139–143. Levitt, M.; Perutz, M. F. *J. Mol. Biol.* **1988**, *201*, 751–754. Thomas, K. A.; Smith, G. M.; Thomas, T. B.; Feldmann, R. J. *Proc. Nat. Acad. Sci. U.S.A.* **1982**, *79*, 4843–4847. Hydrophobicity may also play a role.

(18) Present address: Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA 91125.

Department of Chemistry
Columbia University
New York, New York 10027

Jeffrey R. Bocarsly
Jacqueline K. Barton*,[18]

Received April 5, 1989