Mapping an Antibody Binding Site by Nuclear Decay

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The study of proteins and their complexes by cleavage of polypeptide chains provides a new approach to understanding their behavior in solution.[1−10] All work so far has employed cleaving reagents—metal complexes, proteases, etc.—that may have limited compatibility with the system of interest. We reasoned that radiolabeling a ligand which binds at a specific site on a protein would provide a potential source of bond-breaking energy during the subsequent nuclear decay. This might cleave the complex such that analysis of resulting protein fragments would reveal the immediate surroundings of the radionuclide. We chose isotopic substitution, which does not change the structure of a molecule, so that we could assess the effects of radioactive decay on a structurally characterized complex. Monoclonal antibody CHA255 specifically binds *p*-substituted (*S*)-benzyl-EDTA−[In] chelates (Figure 1).[11−13] Allowing CHA255 to bind a chelate containing the radioisotope ^{111}In ($t_{1/2}$ 67 h) results in chain cleavage at several points within the binding pocket of the antibody.

Two different chelates were prepared with radioactive indium-111 and stable indium, followed by incubation with CHA255. Chelation of 2.17 μM (100 mCi/mL, 3.7 GBq/mL) ^{111}InCl$_3$ (Nordion International, Ontario, Canada) or 2.17 μM stable InCl$_3$ by 1 mM diethylenetriamine pentaacetic acid (DTPA) or (*S*)-[*p*-nitrobenzyl]-EDTA was carried out in 0.1 M sodium citrate at pH 5.0 for 1 h at room temperature (rt).[14]

Cleavage experiments were set up using 1 μM CHA255 in 0.2 M HEPES, 10% glycerol, pH 7.4. Glycerol was included to scavenge reactive species produced by radiolysis of H$_2$O, to minimize *inter*molecular cutting. Four reactions were carried out in parallel: each contained either 0.1 mM (*S*)-[*p*-nitrobenz-yl]-EDTA or DTPA, with either 0.22 μM stable In or ^{111}In. Reactions were incubated at rt for 1.25$t_{1/2}$ and quenched with SDS-PAGE sample application buffer, frozen in liquid N$_2$, and stored at −70 °C. When the ^{111}In had decayed to ~100 kBq, each reaction was analyzed by denaturing gel electrophoresis and electroblotting.

The practical constraints of these experiments are dominated by the need for a sensitive method to detect and identify the tiny amounts of polypeptide fragments produced (≤1 pmol) on a blot containing ^{111}In. Immunostaining with anti-F$_c$ or anti-C$_\lambda$ antibody−alkaline phosphatase conjugates allowed us to visualize the CHA255 heavy or light chain fragments after color development with BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (nitro blue tetrazolium). Because these conjugates bind specifically to regions remote from the CHA255 binding sites, they serve to reveal fragments produced by cleavage within these sites (see Figure 2). We estimated the molecular weights of the fragments by comparing their electrophoretic migration to CHA255 fragments of known residue length, prepared by treatment with trypsin or cyanogen bromide.

Figures 3 and 4 show the effect of ^{111}In decay on antibody CHA255. Incubation with stable indium chelates affords no fragmentation. Radioactive DTPA−[^{111}In] does not bind to CHA255, and ^{111}In decay from this complex causes very little long-range chain cleavage. However, (*S*)-[*p*-nitrobenzyl]-EDTA−[^{111}In] binds to the antibody with a dissociation constant $K \approx 10^{-9}$ M, undergoes radioactive decay, and promotes scission of the protein backbone (lane 3 of Figure 3 and lane 2 of Figure 4). The heavy chain is specifically cut in two locations, yielding C-terminal fragments of ~44 and ~37 kDa; the light chain is cut in several locations, yielding C-terminal fragments with masses ~20, ~15−18, and ~12 kDa.

The crystal structure[13] shows how residues from the six complementarity determining regions (CDRs) interact with the hapten (Figure 1). When bound to CHA255, the benzyl-EDTA-[In] moiety is capped by a histidine side chain (His99H, in CDR H3). This coordination brings the Nϵ of the histidine sidechain within 2.4 Å of the metal, making it the closest amino acid atom to the indium. The ~37 kDa C-terminal fragment observed for the heavy chain is consistent with efficient cleavage of the heavy chain at this location or at the adjacent Arg100H, which hydrogen bonds to the hapten.

In addition, the hapten EDTA−[In] moiety is involved in a hydrogen-bond network with five residues in other regions of CHA255. The observed ~44 kDa heavy chain fragment is consistent with cleavage at the only other heavy chain residue that hydrogen bonds to the hapten, Thr33H in CDR H1. The light chain contains four residues (Tyr34L and Asn36L in CDR L1, Gly52L in CDR L2, and Trp98L in CDR L3) in the hydrogen bond network. Cleavage of the light chain near these residues would produce ~20, ~18, and ~13 kDa fragments. Observed light chain fragments match these values within experimental uncertainty, though there are multiple fragments near 15−18 kDa (Figure 4).

Cleavage at CDR H2 would produce a ~40 kDa fragment, but the yield is insignificant (Figure 3, lane 3). This is surprising, since residues 51H−53H in CDR H2 are as close to the metal as the light chain residues; however, CDR H2 is unique in not being part of the hydrogen bond network to the hapten.

Comparing the intensity of immunostained cleavage products with serially diluted standards, we find the yield of detectable backbone cleavage ranges from ~5 to 30%. At 2.4 Å from the nearest side chain atom (5 Å from the nearest backbone atom), cleavage efficiency is ~30%. At distances of 8−10 Å, effi-ciencies decrease to ~5−10% for the heavy and light chain residues that hydrogen bond to the EDTA−[In]. Since residues 51H−53H have several atoms within 8−10 Å of the metal, lack of cleavage at CDR H2 implies that proximity is not a sufficient condition for backbone cleavage. On the other hand, the 15−18 kDa light chain fragments indicate that cleavage extends beyond CDR L2 into the neighboring framework 3 region.

Indium-111 decays by electron capture—recoiling and emit-ting not only gamma rays but also low energy Auger electrons and an X-ray. Martin and Holmes found that the electron cap-ture decay of bound iodine-125 caused localized strand breaks in DNA.[15] While the chemistry of irradiated solutions of proteins has been studied extensively,[16,17] the details of the extremely short-range molecular events that cause cutting of

(1) Rana, T. M.; Meares, C. F. *J. Am. Chem. Soc.* **1990**, *112*, 2457.
(2) Rana, T. M.; Meares, C. F. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 10578.
(3) Schepartz, A.; Cuenoud, B. J. *J. Am. Chem. Soc.* **1990**, *112*, 3247.
(4) Hoyer, D.; Cho, H.; Schultz, P. G. *J. Am. Chem. Soc.* **1990**, *112*, 3249.
(5) Heyduk, E.; Heyduk, T. *Biochemistry* **1994**, *33*, 9643.
(6) Wu, J. H.; Perrin, D. M.; Sigman, D. S.; Kaback, H. R. *Proc. Natl. Acad. Sci. U.S.A.* **1995** , *92*, 9186.
(7) Ettner, N.; Metzger, J. W.; Lederer, T.; Hulmes, J. D.; Kisker, C.; Hinrichs, W.; Ellestad, G. A.; Hillen, W. *Biochemistry* **1995**, *34*, 22.
(8) Ermacora, M. R.; Ledman, D. W.; Fox, R. O. *Nat. Struct. Biol.* **1996**, *3*, 59.
(9) Grammer, J. C.; Loo, J. A.; Edmonds, C. G.; Cremo, C. R.; Yount, R. G. *Biochemistry* **1996**, *35*, 15582.
(10) Hori, R.; Carey, M. *J. Biol. Chem.* **1997**, *272*, 1180.
(11) Reardan, D. T.; Meares, C. F.; Goodwin, D. A.; McTigue, M.; David, G. S.; Stone, M. R.; Leung, J. P.; Bartholomew, R. M.; Frincke, J. M. *Nature* **1985**, *316*, 265.
(12) Meyer, D. L.; Fineman, M.; Unger, B. W.; Frincke, J. M. *Bioconjugate Chem.* **1990**, *1*, 278.
(13) Love, R. A.; Villafranca, J. E.; Aust, R. M.; Nakamura, K. K.; Jue, R. A.; Major, J. G.; Radhakrishnan, R.; Butler, W. F. *Biochemistry* **1993**, *32*, 10950.
(14) Meares, C. F.; McCall, M. J.; Reardan, D. T.; Goodwin, D. A.; Diamanti, C. I.; McTigue, M. *Analyt. Biochem.* **1984**, *142*, 68.
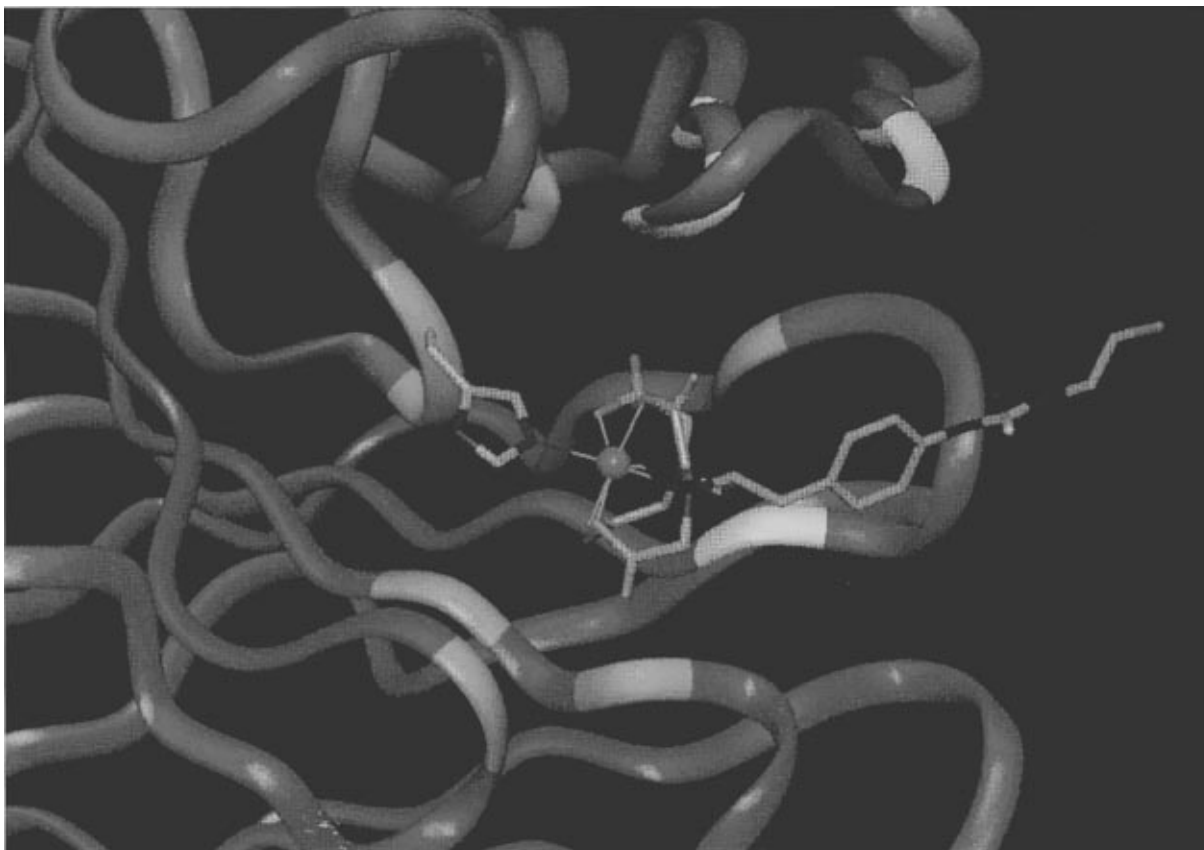
Figure 1. Indium chelate bound to antibody CHA255, from the crystal structure.[13] Heavy chain is blue; light chain is green; chelate is rendered as a stick model with indium a red sphere. Chain segments in red are residues involved in hydrogen bonding to the hapten; these are located in five of the six complementarity-determining regions (CDRs) and correspond to backbone cleavage sites. Segments in yellow are residues within van der Waals contact of the hapten, but not hydrogen bonded; this includes CDR H2 (upper right), which is not cleaved to a significant degree.
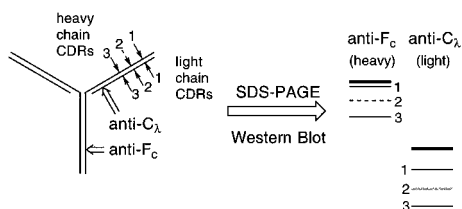

Figure 2. Strategy for detection of backbone cleavage by electroblotting and immunostaining with antibody conjugates that bind to epitopes located between the cleavage sites and the C termini of the heavy and light chains. In the text, heavy chain CDRs 1−3 are referred to as CDR H1, CDR H2, and CDR H3; similarly, light chain CDRs are CDR L1, etc.

the protein backbone within a few angstroms of a decaying nucleus have yet to be elucidated.

Nonetheless, the potential identification of residues within ~10 Å of a radiolabel has a number of possible applications to the study of macromolecules, beyond the rather special complex described here. For example, the large multisubunit protein/nucleic acid complexes involved in gene transcription and translation are of a size and complexity that make their proximity relationships in solution difficult to determine with such resolution. In experiments with a different monoclonal antibody, we have found that the 2.2 MeV β decay of yttrium-90 leads to results similar to those reported here (R. Mogul and C. F. Meares, unpublished results). Also, Rosenthal and Fox found that the 1.7 MeV β decay of phosphorus-32 in one strand of double-stranded DNA cleaved the *opposite* strand with ≈5% efficiency.[18] This is comparable to our results, since a phosphorus atom on one strand of B DNA is within 8−10 Å of the
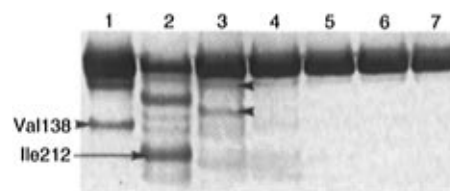

Figure 3. Effects of nuclear decay on CHA255 heavy chain (10% PAGE, immunoblot stained with anti-F_c): Lane 1, C-terminal marker fragment beginning at Val138 (CNBr); lane 2, C-terminal marker fragment beginning at Ile212 (Trypsin); lane 3, (S)-[p-nitrobenzyl]-EDTA−[^{111}In], with arrows showing fragments produced by cleavage near CDR H1 and CDR H3; lane 4, DTPA−[^{111}In]; lane 5, (S)-[p-nitrobenzyl]-EDTA−[In]; lane 6, DTPA−[In]; lane 7, CHA255 only (untreated). Markers were characterized by N-terminal sequencing.
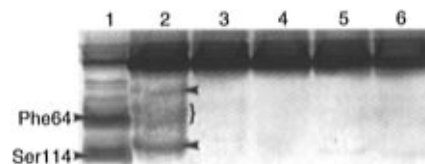

Figure 4. Effects of nuclear decay on CHA255 light chain (15% PAGE, immunoblot stained with anti-C_λ): lane 1, C-terminal marker fragments beginning at Phe64 and Ser114 (Trypsin); lane 2, (S)-[p-nitrobenzyl]-EDTA−[^{111}In], showing fragments produced by cleavage near CDR L1, L2 (bracket), and L3; lane 3, DTPA−[^{111}In]; lane 4, (S)-[p-nitrobenzyl]-EDTA−[In]; lane 5, DTPA−[In]; lane 6, CHA255 only (untreated).

opposite strand. Taken together, these observations suggest that radioisotopes can provide a "reagentless" strategy to map proximities within complex biological systems.

(15) Martin, R. F.; Holmes, N. *Nature* **1983**, *302*, 452.
(16) Garrison, W. M. *Chem. Rev.* **1987**, *87*, 381.
(17) Greiner, D. P.; Hughes, K. A.; Meares, C. F. *Biochem. Biophys. Res. Commun.* **1996**, *225*, 1006.
(18) Rosenthal, P. N.; Fox, M. S. *J. Mol. Biol.* **1970**, *54*, 441.

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