Synchrotron radiolysis of proteins is dependent on the solvent accessibility and reactivity of specific amino acid side chains. Since discrete residues will have altered solvent accessibilities upon interaction of a protein with ligand, synchrotron radiolysis provides a method to probe these changes and, in combination with mass spectrometric analysis, identify the amino residues involved in ligand binding. A change in the hydroxyl radical induced modification of the protein versus the protein–ligand complex is representative of a “footprint” for residues that interact at the binding site. We have applied this method to study the interaction between profilin and poly-L-proline as a model protein-ligand system, since its crystal structure has elucidated the specific amino acid residues in profilin that interact with poly-L-proline. Our studies on the profilin/poly-proline complex highlight a number of issues that must be considered for x-ray footprinting experiments. X-ray footprinting of macromolecular complexes requires that the amino acids forming the binding interface exhibit substantially different solvent accessibilities in the complexed and free states. In addition to surface accessibilities, the kinetics of the protein-ligand complex needs to be considered for x-ray footprinting. The profilin/poly-proline complex has a dissociation constant on the order of 100 µM. Assuming a fast on-rate (10⁶ M⁻¹ S⁻¹), the off-rate can be calculated to be 10⁴ s⁻¹, and therefore, the half-life for this complex is 70 µs. Thus, off rates need to be carefully considered when designing experiments. Based on these criteria for protein footprinting established by our profilin/poly-L-proline analysis, we are extending our studies to other proteins-ligand complexes that maintain high affinities and substantial surface accessibility changes.