Specific Cleavage of a Protein by an Attached Iron Chelate

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Proteins and nucleic acids carry out specific functions, such as molecular recognition, information transfer, and catalysis, which depend in detail on the structure of each macromolecule. Cleavage of DNA or RNA by metal chelates is an important new approach to characterizing structural features of nucleic acids and their complexes in solution,[1,2] because chain scission by reactive oxygen species occurs close to where the redox-active metal complex is bound. Likewise, cleavage of a protein by a reagent bound at a particular site could give information about the folding of the polypeptide chain.[3]

For a protein cleavage method, several criteria must be met. Reagents capable of cleaving peptide bonds and suitable for attachment at specific sites must be devised. If peptides are to be identified by standard sequencing procedures, conditions must be found under which cleavage of the polypeptide backbone predominates over degradation of amino acid side chains, producing fragments with free amino and carboxyl termini. Cleavage reactions should occur at or near physiological conditions of pH, temperature, and ionic strength. Generally, it will be preferred that the chain not be cleaved at the amino acid residue carrying the cleaving reagent but rather at sites that are nearby in the three-dimensional structure (though possibly distant in the primary sequence).

We have achieved this by introducing a metal-binding site at one position in a polypeptide chain. We attached an iron chelate to a unique residue on the protein bovine serum albumin (BSA), treated the protein–chelate conjugate with H_2O_2/ascorbate, and observed the production of three peptide fragments which together account for the entire polypeptide chain.

The experimental procedure is outlined in Figure 1. Site-specific conjugation of BSA was achieved by alkylation of the only free sulfhydryl (cysteine-34)[4] with 1-(*p*-bromoacetamidobenzyl)-EDTA.[5] The loss of –SH was monitored with Ellman's reagent,[6] and it matched the gain in chelating groups, measured by $^{57}Co^{2+}$ titration.[7] This indicates that the reaction was restricted to the free cysteine residue of the protein. The chelating groups were specifically loaded with Fe^{2+}, and cleavage was accomplished by a 10-s treatment with ascorbate and H_2O_2 at pH 7.0, 25 °C.

The products were analyzed by electrophoresis.[9] In Figure 2 (lanes 5–9), three major cleavage products may be observed, at positions corresponding to molecular weights ≈ 45K, 17K, and 5K. Control experiments demonstrated the specificity of the observed chain scission and its dependence on protein tertiary structure. Without the chelate, but with free Fe^{2+} in the reaction medium, only a faint band at M_r < 45K was produced (lane 3). No significant cleavage was observed when the protein was unfolded (0.5% sodium dodecyl sulfate or 6 M guanidinium chloride) or in the presence of an *unattached* chelate (2 mM 1-(*p*-nitrobenzyl)-EDTA-Fe, 20 mM ascorbate, 5 mM H_2O_2) (data not shown).

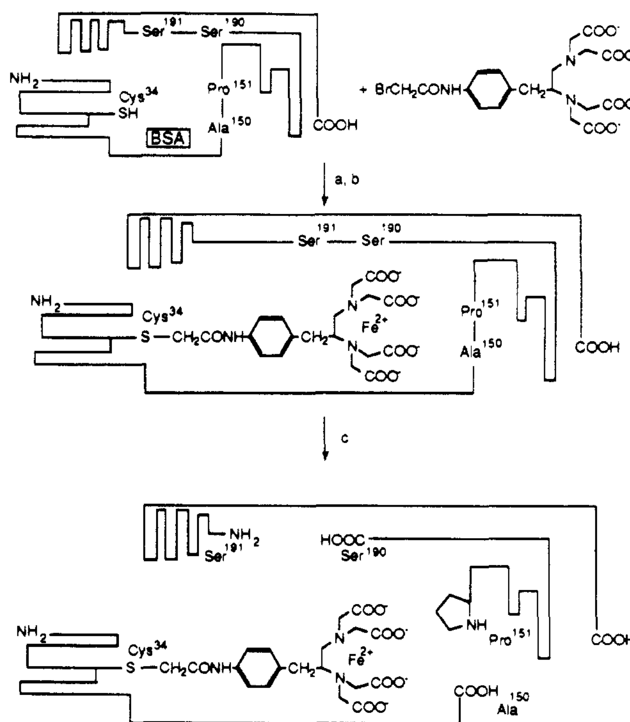

Figure 1. (a) Conjugation: 1.1 mM BSA, 2.2 mM 1-(*p*-bromoacetamidobenzyl)-EDTA, 0.1 M phosphate, pH 8.0, 37 °C, 5 h. (b) Iron loading: 20 μL of freshly prepared 10 mM $FeSO_4$ added to 100 μL of 0.81 mM conjugated BSA (0.1 M phosphate, pH 7.0) and allowed to stand at room temperature for 25 min. Excess iron scavenged by adding 50 μL of 20 mM EDTA (0.1 M phosphate, pH 7.0) and separated by spin-column gel filtration chromatography.[8] (c) Polypeptide chain scission: at 25 °C, 4 μL of 0.58 mM conjugated BSA in 0.1 M phosphate buffer, pH 7.0, was mixed with 20 μL of 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (Hepes) buffer, pH 7.0, containing 20 mM sodium ascorbate and 5 mM hydrogen peroxide. After ≈ 10 s, the reaction was stopped by adding 35 μL of sodium dodecyl sulfate sample application buffer[9a] and 0.1 M thiourea.[1c]

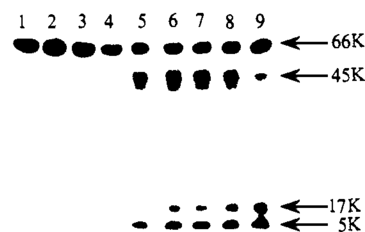

Figure 2. Separation of the cleavage products by sodium dodecyl sulfate–polyacrylamide gel electrophoresis: unmodified BSA (lane 1); BSA with 20 mM ascorbate and 5 mM H_2O_2 (lane 2) or with 20 mM ascorbate, 5 mM H_2O_2, and 1 mM $FeSO_4$ (lane 3); chelate-conjugated BSA in the absence of ascorbate/H_2O_2 (lane 4); cleavage of chelate-conjugated BSA in the presence of ascorbate/H_2O_2, quenched with 0.1–0.5 M thiourea (lanes 5–9).

The cleaved peptides were identified by their N- and C-terminal sequences.[10] The N-terminal sequence of the cleaved 17K peptide is Asp-Thr-His-Lys-Ser-, identical with the N-terminal sequence of native BSA.[4] The C-terminal sequence of the 17K peptide is -Tyr-Phe-Tyr-Ala, which is uniquely located at residues 147–150 of BSA.[4] Thus, scission occurred after residue Ala-150, producing a 150 amino acid fragment having M_r 17441 and containing the N-terminus of BSA.

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(10) N-terminal sequences were determined by using an Applied Biosystems 470A gas-phase sequencer. Carboxyl termini were identified by carboypeptidase Y digestion (Kitabatake, N.; Indo, K.; Doi, E. *J. Agric. Food Chem.* **1988**, *36*, 417–420).

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The N-terminal sequence of the 5K peptide is Pro-Glu-Leu-Leu-Tyr-Ala-, which is uniquely located at residues 151-156 of BSA.[4] The C-terminus of the 5K peptide is -Lys-Val-Leu-Thr-Ser, uniquely located at positions 186-190.[4] Thus the 5K peptide was produced by two peptide bond cleavages: one between Ala-150 and Pro-151, and the other after Ser-190, giving a 40 amino acid peptide having M_r 4535.

The N-terminal sequence of the 45K fragment is Ser-Ala-Arg-Gln-Arg-Leu-, which is uniquely located at residues 191-196.[4] The C-terminal sequence of the 45K fragment is -Gln-Thr-Ala-Leu-Ala, identical with the *C*-terminal sequence of native BSA.[4] Thus, cleavage occurred between residue Ser-190 and Ser-191, giving a 392 amino acid fragment having M_r 43821 and containing the C-terminus of BSA.

Remarkably, the amino acids at the sites of cleavage are detected unaltered; the sequences of the fragments exactly match that of the parent protein, with no gaps. For standard sequencing procedures, the cleavage has the same result as cleavage by a proteolytic enzyme. However, the *yield* of the N-terminal amino acid in the first cycle of Edman degradation is roughly half that of the following residue obtained in the second cycle, implying that other reactions besides hydrolysis accompany the production of the new N-terminus. The extent of degradation of amino-acid side chains was explored by comparing the experimentally determined amino acid composition of one cleaved peptide (17K) with the expected composition.[11] Loss of natural amino acids was slight; in the worst case, the histidine content was 83% of theoretical.

The observed cleavage at both Ala-Pro and Ser-Ser shows that this process does not depend strongly on the chemical reactivity of the amino acid residue which is to be cleaved. Since the peptide bond between the other Ser-Ser sequence in the protein (Ser-270 and Ser-271) was not affected, cleavage appears to depend on proximity of the reagent to the polypeptide chain (no crystal structure is available for comparison).

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(11) The 17K peptide was hydrolyzed in 6 N HCl for 24 h at 110 °C. Trp, Cys, and Met were not determined.

Electrochemical Synthesis of Ultrathin-Film Composite Membranes

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Membrane-based separations are often less energy intensive and more resource conservative than alternative separation methods.[1] Synthetic membranes have, therefore, been the focus of considerable recent research effort.[1] The development of ultrathin-film composite membranes was one of the most important breakthroughs in the synthetic-membrane area;[1b,2] these membranes consist of a porous support layer and a dense, ultrathin[2] active layer. The porous support layer provides mechanical strength yet is highly permeable. The separation process occurs primarily in the ultrathin active layer; because this layer is thin, the overall flux of permeate through the membrane is high. Thus, composite membranes can provide good mechanical strength, high selectivity, and high permeability. This combination of attributes usually cannot be obtained with homogeneous membranes.[1b,3]

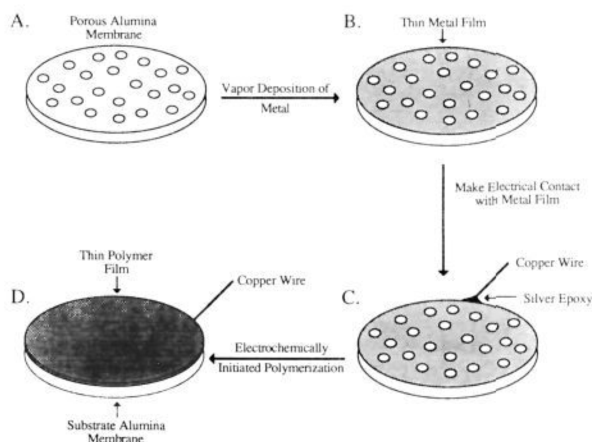

Figure 1. Schematic of ultrathin-film composite membrane fabrication procedure.

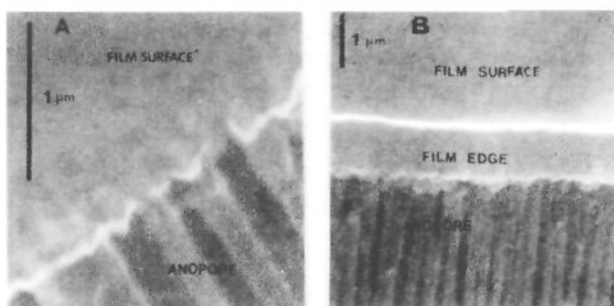

Figure 2. Electron micrographs of cross sections of two ultrathin-film composite membranes.

We have developed a new method for preparing ultrathin-film composite membranes. This method involves electrochemically initiated polymerization at a microporous support-membrane surface and yields an ultrathin[2] polymer film on one face of the support membrane. We describe the synthesis, and preliminary results of electrochemical characterization, of such membranes in this communication.

Figure 1 shows a schematic of the procedure used to prepare the ultrathin-film composites. Anopore (Alltech) Al_2O_3 filters were used as the support membranes;[4] Anopore is 65% porous and contains linear, cylindrical, 200-nm-diameter pores. The Anopore surface is first coated with a ca. 50-m layer of gold;[5] this layer is too thin to block the pores (Figure 1B). A copper wire is attached, and the resulting electrode (Figure 1C) is immersed into a solution containing the electropolymerizable monomers. Electropolymerization causes a thin polymer skin to "grow" over the Anopore surface (Figure 1D).

Our work to date has focused on copolymers of divinylbenzene (DVB) and ethylvinylbenzene (EVB). These monomers can be reduced electrochemically to anions which polymerize via a conventional anionic mechanism.[6] Technical grade DVB (55% DVB and 45% EVB, Polysciences) was extracted with 10% NaOH to remove polymerization inhibitors. The extract was washed three times with purified water. The DVB/EVB was then passed through a column of activated alumina; the effluent was stored (in the dark) over calcium hydride at −5 °C. Polymerization solutions were prepared by mixing measured volumes of the purified DVB/EVB with *N,N*-dimethylformamide (DMF);[7] solutions

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