Hydroxylapatite Chromatography of Protein-Sodium Dodecyl Sulfate Complexes

A NEW METHOD FOR THE SEPARATION OF POLYPEPTIDE SUBUNITS

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SUMMARY

Hydroxylapatite (calcium phosphate) chromatography was investigated as a method for separating protein subunits dissociated with sodium dodecyl sulfate (SDS). The proteins used included: a series of 11 well characterized proteins composed of single or identical subunits varying in molecular weight from 11,700 to 165,000; hemoglobin, composed of α and β chains of 146 and 141 amino acids; and vaccinia virus structural proteins, composed of more than 15 different polypeptide subunits. Reduced proteins were complexed with SDS and adsorbed to columns of hydroxylapatite. Using linear gradients of sodium phosphate, pH 6.4, containing 0.1% SDS and 1 mM dithiothreitol, all tested proteins eluted between 0.2 and 0.5 M phosphate. A useful feature of the method is that proteins do not all elute in order of molecular weight. Thus, polypeptide separations on hydroxylapatite are different from those obtained by polyacrylamide gel electrophoresis or gel filtration in SDS. The high resolution of the method was demonstrated by separating the α and β chains of hemoglobin and complex mixtures of viral polypeptides. By using polyacrylamide gel electrophoresis and hydroxylapatite chromatography in succession, it has been possible to separate vaccinia virus structural polypeptides that had not previously been resolved.

EXPERIMENTAL PROCEDURE

Materials—Hydroxyapatite (Bio-Gel HT) was obtained from Bio-Rad Laboratories; sodium dodecyl sulfate was the special grade from Mann and dithiothreitol came from Calbiochem. All other chemicals were reagent grade.

Rabbit globin, labeled with 14C histidine, was a gift of Dr. W. French Anderson. Vaccinia virus proteins, labeled with 3H- or 14C-amino acids were prepared as previously described (1). Cytochrome c (horse heart), myoglobin (sperm whale), chymotrypsinogen A (beef pancreas), and bovine serum albumin were obtained from Mann. Carbonic anhydrase (beef blood), glyceraldehyde phosphate dehydrogenase (rabbit muscle), and catalase (beef liver) came from Worthington; lysozyme, thyroglobulin (bovine), and human hemoglobin came from Calbiochem, Sigma, and Pentex, respectively.

Preparation of Protein-SDS Complexes—Radioactively labeled proteins were dissolved in 0.01 M sodium phosphate, pH 6.4-1% SDS-1% mercaptoethanol and placed in a boiling water bath for 2 min. The samples were then dialyzed 10-fold with 0.01 M sodium phosphate, pH 6.4-0.1% SDS and applied directly to hydroxylapatite columns. Unlabeled proteins, at a concentration of 4 mg per ml, were complexed with SDS in a similar manner except that 2% SDS and 2% mercaptoethanol were used and the samples were dialyzed overnight instead of being diluted.

Chromatography—Hydroxylapatite was washed with 0.01 M sodium phosphate, pH 6.4-0.1% SDS-1 mM DTT. Columns (0.9 x 20 cm) were poured over a 0.5-cm layer of fine grade Sephadex G-25. Protein samples, from 1 to 30 ml in volume, were applied and washed in with 1 to 2 column volumes of the 0.01 M phosphate DTT solution. Linear gradients were formed with a simple two-chambered device and flow rates of 5 to 15 ml per hour were obtained by gravity. Sixty drop frac-
Separation of α and β Hemoglobin Chains—The α and β chains of rabbit hemoglobin, which are 146 and 141 amino acids long, were separated by hydroxylapatite chromatography. Rabbit globin, labeled with [14C]histidine, was reduced with mercaptoethanol and complexed with SDS. The globin-SDS complexes were completely adsorbed to hydroxylapatite when applied in 0.1% SDS-0.1 mM DTT-0.01 M sodium phosphate, pH 6.4-1 mM DTT. When polyacrylamide gel electrophoresis was to be followed by hydroxylapatite chromatography, the pooled fractions were made 1% in mercaptoethanol, heated at 100° for 2 min, allowed to cool to room temperature, and applied directly to hydroxylapatite columns.

RESULTS

Separation of α and β Hemoglobin Chains—The α and β chains of rabbit hemoglobin, which are 146 and 141 amino acids long, were separated by hydroxylapatite chromatography. Rabbit globin, labeled with [14C]histidine, was reduced with mercaptoethanol and complexed with SDS. The globin-SDS complexes were completely adsorbed to hydroxylapatite when applied in 0.1% SDS-0.1 mM DTT-0.01 M sodium phosphate, pH 6.4-1 mM DTT. The peak fractions in Fig. 1 were pooled, dialyzed against 0.01 M sodium phosphate, pH 6.4-0.1% SDS-0.1% mercaptoethanol and chromatographed on new hydroxylapatite columns as in Fig. 1. A, Fractions 70 to 75 from Fig. 1; B, Fractions 82 to 88 from Fig. 1.

Separation of Vaccinia Virus Structural Polypeptides—Vaccinia virus contains a large number of structural polypeptides which are incompletely resolved by polyacrylamide gel electrophoresis in SDS (9, 10). [14C]Amino acid labeled vaccinia virus structural polypeptides were completely adsorbed to hydroxylapatite. Upon elution with a 0.2 to 0.5 M phosphate gradient containing SDS and DTT, eight peaks were resolved (Fig. 3). Preliminary experiments with a 4-cm column gave poorer resolution. The pooled fractions, indicated by Roman numerals I to VI (Fig. 3), eluted in the same order when re-chromatographed. The vital polypeptides fractionated by hydroxylapatite
FIG. 4. Polyacrylamide gel electrophoresis of 14C-amino acid-labeled vaccinia virus structural polypeptides fractionated by hydroxylapatite chromatography. Pooled fractions from Fig. 3 were dialyzed overnight against water, lyophilized, dissolved in SDS and mercaptoethanol, mixed with total 3H-amino acid-labeled vaccinia virus structural polypeptides, heated at 100°C for 2 min, and subjected to electrophoresis on 10% polyacrylamide gels. The gels were sliced and counted as described. The Roman numerals refer to the fractions pooled in Fig. 3.

The separation of polypeptides with similar electrophoretic mobilities by hydroxylapatite chromatography was directly demonstrated in the following experiments. Vaccinia virus polypeptides, labeled with [3H]tryptophan and [14C]-amino acids were separated by polyacrylamide gel electrophoresis into three molecular weight groups designated by A, B, and C (Fig. 5). Upon chromatography, the polypeptides with lowest electrophoretic mobility (A) eluted as Peak VI; the polypeptides with intermediate mobilities (B) eluted as Peaks II, V, and VI; and the most rapidly migrating polypeptides (C) eluted as Peaks I, II, III, and IV (Fig. 6). Differences in 3H:14C ratios suggest that the separated polypeptides have different amino acid compositions. A appears to be composed of unresolved polypeptides with different contents of tryptophan. Although viral polypeptides separated quite differently by hydroxylapatite chromatography and polyacrylamide gel electrophoresis, polypeptides with lower electrophoretic mobilities tended to elute at higher molarities of phosphate.

Chromatography of Proteins with Identical Subunits—A series of well characterized proteins, thought to contain 1 or more identical subunits, was examined by hydroxylapatite chromatography in SDS (Fig. 7). Most eluted as a single major peak, although bovine serum albumin and ovalbumin appeared somewhat heterogeneous. All of the proteins eluted between 0.2 M
and 0.5 mM phosphate and no correlation with molecular weight was found. For example, polypeptides ranging in molecular weight from 11,700 (cytochrome c) to 165,000 (thyroglobulin) eluted at lower molarities of phosphate than serum albumin, which has a molecular weight of 68,000.

Lysozyme, serum albumin, and ovalbumin, which are composed of single polypeptide chains, were also chromatographed without SDS. All eluted at less than 0.15 M phosphate, indicating that these proteins bind less strongly to hydroxylapatite in the absence of SDS.

**DISCUSSION**

These studies demonstrate the usefulness of hydroxylapatite chromatography in SDS as a method for analytical and preparative separations of protein subunits. Since polypeptides are not all eluted from hydroxylapatite in order of molecular weight, the separations are different from those obtained in SDS by polyacrylamide gel electrophoresis or gel filtration. The resolving power of SDS-hydroxylapatite columns was demonstrated by separating the α and β chains of hemoglobin and complex mixtures of viral polypeptides. By using polyacrylamide gel electrophoresis and hydroxylapatite chromatography in succession, vaccinia virus structural polypeptides that had not previously been resolved were separated. During the past year we have used hydroxylapatite chromatography for a number of other problems, including the separation of SDS-dissociated membrane glycoproteins, with equally good results. Recoveries of proteins were quantitative even with microgram amounts of radioactively labeled materials. The precise elution molarity of proteins appeared to depend somewhat on sample load, column length, and steepness of the gradient. Moreover, we found that proteins denatured with urea are less well retained or not retained at all by hydroxylapatite columns equilibrated with 1 mM phosphate buffers. This was attributed to a decrease in the number of carboxyl groups on the surface of proteins in the random coil configuration (13). In contrast we find that proteins denatured with SDS bind more strongly than native proteins to hydroxylapatite. The difference in adsorption of proteins in urea and SDS to hydroxylapatite may be explained by the very different states of the proteins in the two types of solvents. At sufficiently high monomer concentrations of SDS (greater than $5 \times 10^{-4}$ M) there is a wide variety of proteins bind identical amounts of SDS (3). A complex with a stoichiometry of 0.4 g of SDS per g of protein is formed between 5 and $8 \times 10^{-4}$ M SDS monomer (3). A second complex, which is saturated at 1.4 g of SDS per g of proteins is observed above $8 \times 10^{-4}$ M SDS monomer (3). The saturated protein-SDS complexes exist as rod-like molecules, the length of which varies uniquely with the molecular weight of the protein moieties (4).

Presumably, the large amount of SDS bound or the altered conformation of the protein is responsible for the strong attachment to hydroxylapatite. In aqueous solutions SDS exists as monomer and micellar aggregates, but only the monomer form binds to proteins (3). We considered that the amount of SDS bound to proteins would decrease at phosphate concentrations above 0.2 M since the equilibrium monomer concentration of SDS is dependent on ionic strength (3, 14). All proteins tested eluted from hydroxylapatite at phosphate concentrations between 0.2 and 0.5 M. Although it is clear that proteins are not all eluted in order of molecular weight, the factors responsible for protein separations are not understood. It is possible that at low SDS monomer concentrations, differential binding of SDS or differences in the conformations of proteins become significant.

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