RecA Protein Rapidly Crystallizes in the Presence of Spermidine: A Valuable Step in Its Purification and Physical Characterization†

Jack Griffith* and Carol Getker Shores

Lineberger Cancer Research Center and Departments of Microbiology and Immunology and of Biochemistry, University of North Carolina Medical School, Chapel Hill, North Carolina 27514
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ABSTRACT: The RecA protein of *Escherichia coli*, whether pure or in a crude cell lysate, will rapidly form small crystals (microcrystals) in the presence of low concentrations of spermidine. We describe the conditions of time, pH, and polyamine concentration over which crystallization occurs. Microcrystal formation is inhibited by concentrations of chloride over 25 mM and concentrations of phosphate or sulfate ions as low as 2 mM. Crystallization is not inhibited by high concentrations of other proteins, and the RecA protein microcrystals are easily collected by brief centrifugation. This provides a powerful purification step with high yield. Using this novel property, we prepared over 200 mg of RecA protein at least 95% pure with a single-strand DNA-dependent ATPase activity of 98% from 65 g of cells in 2–3 days. Spermidine was easily removed from the RecA protein by dialysis.

In *Escherichia coli*, the events of general recombination, the induction of the SOS response, and some aspects of DNA repair depend on the functioning of the product of the recA gene RecA protein. The complex enzymatic activities of this protein include stimulation of the proteolytic cleavage of λ and Lex A repressors (Craig & Roberts, 1981; Little et al., 1980; Little, 1984) and an ATP-dependent catalysis of synapse and strand exchange between two homolgous DNA partners in vitro (McEntee et al., 1979; Das Gupta et al., 1981; Radding, 1981; West et al., 1981).

RecA protein binds to single-stranded deoxyribonucleic acid (ssDNA)[1] and to duplex DNA to form helical nucleoprotein filaments as seen by electron microscopy (DiCapua et al., 1982; Dunn et al., 1982; Flory & Radding, 1982). These helical nucleoprotein filaments provide the DNA–protein scaffolds upon which strand exchange occurs. Many factors have been found that stimulate or inhibit their formation including SSB protein, RNA, ATP, and ADP. Because the reactions are often carried out for 1–2 h at 37 °C, the results could be greatly affected by the unknown presence of these factors. We have found that the long filaments observed by EM when apparently pure preparations of RecA protein are incubated with Mg^{2+} and the ATP analogue ATPγS result in part from contamination by small amounts of RNA present in the protein preparation and in part from poly(A) synthesized de novo during the incubation by contaminating polynucleotide phosphorylase (Register and Griffith, submitted for publication). Thus, purification of RecA protein to homogeneity while at the same time preserving its many activities is an important but not trivial problem.

Numerous purifications of RecA protein have been published on the basis of classic chromatographic steps and/or its affinity for ssDNA and subsequent release by ATP (Roberts et al., 1979; Shibata et al., 1979; Weinstock et al., 1979; Cox et al., 1981; Kuramitsu et al., 1981; Cotterill et al., 1982). Even so, any new rapid and powerful purification step would be a welcome addition to the battery of methods available for the purification of this multifunctional protein.

An observation in this laboratory that spermidine will induce RecA protein to rapidly form small crystals may provide such a purification step. We report here the parameters of time, salt content, and polyamine concentration under which crystal formation occurs. Because this appears to be highly unique for the RecA protein, microcrystal formation provides a very powerful purification step with high recovery. A purification scheme taking advantage of this unusual phenomenon is presented, and several others are discussed. In addition, spermidine-induced crystallization may provide a useful tool in searching for *E. coli* proteins that bind to RecA protein, and the crystals themselves, albeit very small, may be valuable in physical studies of RecA protein structure.

Experimental Procedures

Bacterial Cells. RecA protein was prepared from an overproducing strain (*E. coli* KM4104/pDR1453) kindly provided by Dr. A. Sancar. The cells were grown in a Labline high-density fermentor to an optical density of 10 (at 560 nm), and naladixic acid was added to 50 μg/mL for 1 h. The cells were harvested, suspended in 10% sucrose and 0.05 M Tris–acetate (pH 7.5) (lysis buffer), frozen dropwise in liquid nitrogen, and stored at −70 °C.

Purification of RecA Protein. The first steps follow those described by Cox et al. (1981). All steps were carried out at 4 °C. Sixty-five grams of frozen cells was thawed and lysis buffer added to a final volume of 200 mL. Following lysis, centrifugation yielded fraction 1 (9500 mg of protein). The subsequent steps of Polymin-P precipitation, back-washing, and precipitation of the protein with ammonium sulfate were carried out as described (Cox et al., 1981). The protein was resuspended in 50 mL of buffer B plus 0.2 M NaCl and dialyzed overnight against two changes of 4 L of this buffer. The large protein precipitate, which contained no RecA protein, was removed by centrifugation and discarded. The su-

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*Address correspondence to this author at the Lineberger Cancer Research Center, University of North Carolina Medical School.

[1] Abbreviations: ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); EM, electron microscopy; ssDNA, single-stranded deoxyribonucleic acid; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

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pernatant (50 mL, fraction 2) contained 2100 mg of protein.

Fraction 2 was passed over a 2.5 × 50 cm column of phosphocellulose P-11 (Whatman) in buffer B plus 0.2 M NaCl at a rate of 1 mL/min. The protein flow-through was determined by OD_{280} and then pooled, yielding fraction 3 (72 mL, 1100 mg of protein). Fraction 3 was dialyzed for 18 h against 2–4-L changes of buffer A. For optimal spermidine-induced crystallization, it was important not to precipitate fraction 3 with ammonium sulfate and to use a sufficiently large phosphocellulose column to remove all traces of Polymin-P.

To fraction 3 was added 0.5 M spermidine acetate, pH 7.5 [prepared by titration of spermidine free base (Sigma) with acetic acid], to a final concentration of 7 mM. The protein microcrystals were immediately obvious as a white precipitate. The suspension was left on ice for 30 min, and the RecA protein crystals were collected by centrifugation for 15 min at 10000*g*. This yielded 240 mg of RecA protein (fraction 4). Normally the purification was to be carried no further. In this case, the protein was resuspended in buffer A containing 20% glycerol and dialyzed against this buffer for several hours before being frozen in aliquots and stored at −70 °C.

For further purification (illustrated here), the RecA protein microcrystals above were resuspended in 20 mL of buffer C and allowed to dissolve by stirring on ice for 30 min. Half (10 mL, 120 mg of protein) at a time was applied to a 3.2 × 42 cm column of Sephacryl S-200 (Pharmacia), and the protein was passed through the column at a flow rate of 20 mL/h in buffer C. Protein-containing fractions were located by OD_{280}. The ssDNA-dependent ATPase activity of these fractions was determined, the peak fractions were pooled, and the protein was precipitated by addition of solid ammonium sulfate to 0.40 g/mL. The mixture was stirred for 2 h, the protein precipitate was collected by centrifugation as above, suspended in 20 mL of buffer A, and dialyzed overnight against 4 L of buffer A (fraction 5; 210 mg from two filtrations).

For the final chromatographic step, fraction 5 was applied to a 3.2 × 40 cm column of PBE-94 (Pharmacia) which had been equilibrated with buffer A. The column was washed (100 mL/h) with 100-mL volumes of buffer A alone, followed by washes of buffer A supplemented with 0.2, 0.4, and 0.6 M NaCl. The RecA protein eluted sharply with the 0.6 M wash. The protein (fraction 6; 200 mg total) was precipitated with ammonium sulfate (0.4 g/mL), resuspended in buffer A with 20% glycerol, and dialyzed against this buffer overnight prior to freezing and storage as described above.

Acrylamide Gel Electrophoresis. Protein samples were analyzed on 7.5% SDS–acrylamide gels as described by Laemmli (1970) using a Tris–glycine–SDS buffer. Electrophoresis was for 4 h at 125 V. The protein was visualized by silver staining as described by Merril et al. (1981).

ATPase Determinations. Phosphate liberated by the ssDNA-dependent ATPase activity of RecA protein was measured by a modification of the method described by Brown (1982).

Buffers. Buffer A is 0.02 M Tris–acetate (pH 7.5), 10% glycerol, 1 mM DTT, and 0.1 mM EDTA. Buffer B is 20 mM potassium phosphate (pH 6.8), 10% glycerol, 1 mM DTT, and 0.1 mM EDTA. Buffer C is 0.05 M Tris–acetate (pH 7.5), 1 mM DTT, 0.3 M ammonium sulfate, 10% glycerol, and 1 mM EDTA.

RESULTS

Observation of RecA Protein Microcrystals by Electron Microscopy. By chance, we noted that inclusion of 1–10 mM spermidine acetate (pH 7.5) to RecA protein in 10 mM Tris–acetate (pH 7.5) led to the appearance of fibrous microcrystals as seen by EM (Figure 1A). These appeared rapidly, even at RecA protein concentrations as low as 1 μg/mL. The microcrystals measured approximately 0.5 μm × 2 μm, were associated into networks, and showed a fine regular staining pattern over their surface (Figure 1B). Thin-sectioning EM indicated that the crystals have an octagonal cross section (not shown).

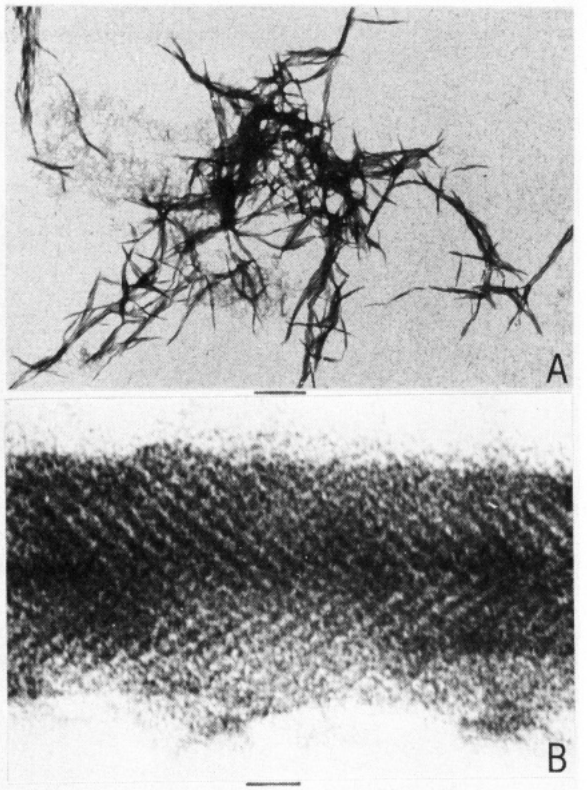

FIGURE 1: RecA protein microcrystals visualized by electron microscopy. RecA protein (10 μg) in 1 mL of 10 mM Tris–acetate (pH 7.5) was induced to form microcrystals by the addition of spermidine acetate (pH 7.5; 7 mM final concentration). Following incubation for 10 min at 20 °C, a drop of the sample was adsorbed to a thin carbon film supported by a copper mesh grid and then stained with 1% aqueous uranyl acetate. The lower magnification field (A) reveals the tendency to form networks. At higher magnification (B), the regular substructure can be seen. Bars equal 1.0 (A) and 0.01 μm (B).

Parameters Affecting RecA Crystal Formation. We show here that RecA microcrystal formation proceeds rapidly in the presence of spermidine or spermine and is inhibited by NaCl concentrations greater than 25 mM and concentrations of SO_4 or PO_4 as low as 2 mM. Because the formation of RecA protein microcrystals led to visible turbidity, measurement of the light-scattering properties of RecA protein solutions after addition of spermidine provided a convenient assay for microcrystal formation. In Figure 2, we show the increase in the optical density at 320 nm with time of a solution containing 260 μg/mL purified RecA protein in a buffer of 10 mM Tris–acetate (pH 7.5) when spermidine chloride was added to 7 mM. Unless noted, all experiments were carried out on ice. (The increase in turbidity at room temperature was at least as fast as that shown here.)

Spermidine-induced microcrystal formation was found to be very sensitive to the anion environment. To study this phenomenon, spermidine free base was titrated with hydrochloric, acetic, or sulfuric acid or sodium phosphate (monobasic), and each of these preparations was buffered with

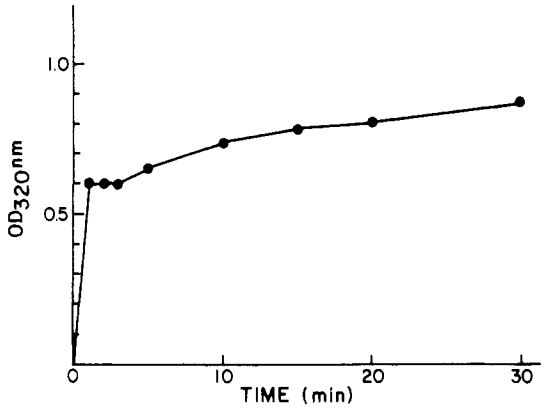

FIGURE 2: RecA protein crystals form rapidly as measured by turbidity at 320 nm. Shown here is the increase in optical density at 320 nm following the addition of spermidine chloride (pH 7.5) to a solution containing RecA protein at 60 μg/mL in 20 mM Tris-HCl (pH 7.5).

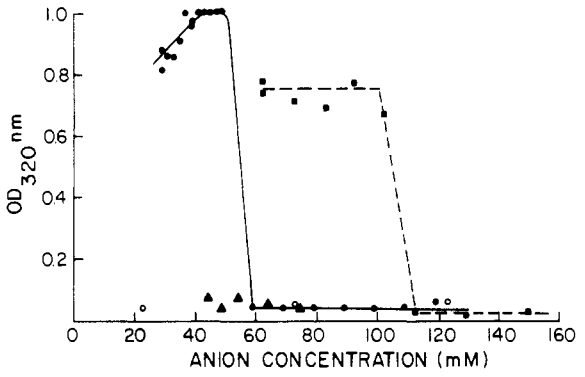

FIGURE 3: Spermidine-induced microcrystallization of RecA protein is sharply inhibited above a critical anion concentration. Samples of RecA protein (260 μg/mL) in 20 mM Tris–acetate (pH 7.5) were mixed with increasing amounts of sodium acetate (■) and incubated with 7 mM spermidine acetate for 30 min on ice, and the optical density was measured at 320 nm. In parallel experiments, chloride (●), phosphate (▲), or sulfate (○) was used as the counterion. Anion concentrations include contributions from all sources.

Tris-HCl, Tris–acetate, Tris–sulfate, or phosphate buffers at pH 7.5, respectively. Each system contained an intrinsic anion concentration, required to maintain the pH. The effect of including additional sodium salts to each system is shown in Figure 3. The sulfate and phosphate systems did not support microcrystal formation. Added NaCl above 25 mM inhibited microcrystallization as did sodium acetate above 40 mM (in the acetate system). Inhibition of microcrystal formation, when it occurred, was very abrupt. In the chloride system, added sodium sulfate or phosphate at 2 mM inhibited crystal formation; in the acetate system, sodium chloride or sulfate inhibited at 25 mM and sodium phosphate at 5 mM (data not shown). Thus, the acetate system was most tolerant of minor anion contaminants.

Spermidine is a member of a family of small polycations, which include putrescine and spermine. Figure 4 shows the effect of increasing concentrations of the chloride salts of these polycations on a solution of RecA protein. Spermidine and spermine induced microcrystal formation, but putrescine did not. Microcrystal formation was inhibited when sufficient spermidine chloride was added to raise the total concentration of chloride to 60 mM. Spermine-induced microcrystallization was not observed at concentrations of NaCl of 0.11 M or higher. Increasing concentrations of spermidine acetate yielded similar shaped curves with a peak at 7 mM (data not shown). In the ranges of 5–15 mM spermidine and 2–15 mM spermine, greater than 95% of the RecA protein was in the form of

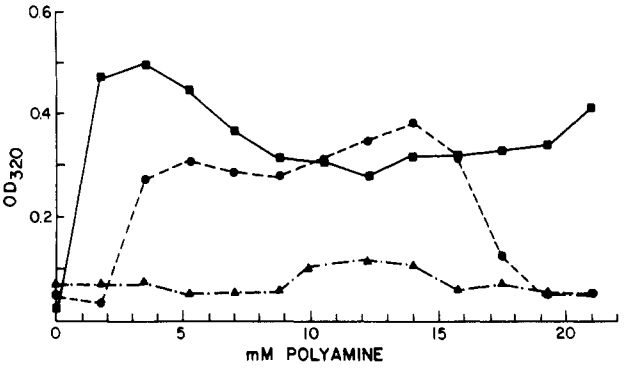

FIGURE 4: RecA protein microcrystallization is promoted by spermidine and spermine but not putrescine. RecA protein (260 μg/mL) in 20 mM Tris-HCl (pH 7.5) was mixed with increasing concentrations of spermidine chloride (pH 7.5) (●), spermine chloride (pH 7.5) (■), or putrescine chloride (pH 7.5) (▲) for 30 min on ice and microcrystallization followed by measurement of the optical density at 320 nm.

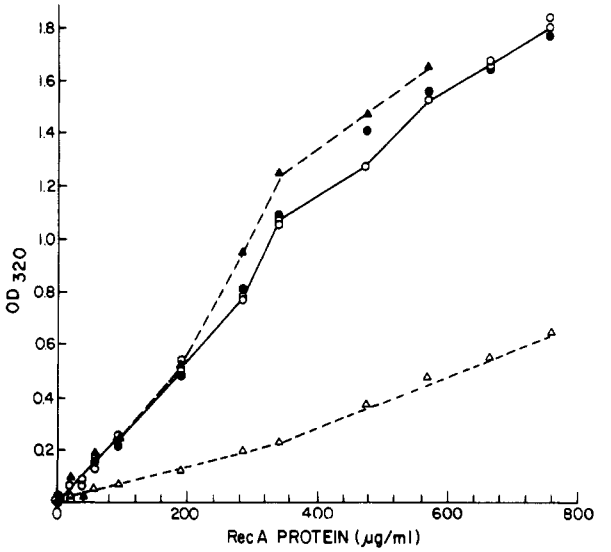

FIGURE 5: Turbidity due to RecA protein microcrystallization depends on the concentration of RecA protein and spermidine but is independent of other proteins. Spermidine acetate (pH 7.5) was added at a concentration of either 7 (▲, ●, ○) or 0.7 mM (△) to solutions containing from 10 to 800 μg/mL RecA protein in 20 mM Tris–acetate (pH 7.5). After incubation for 30 min on ice, the optical density was measured at 320 nm. Duplicate points at 7 mM spermidine are indicated as open and filled circles. In a third set (▲), bovine serum albumin was added to 1 mg/mL in each sample.

microcrystals that could be collected by brief centrifugation. Thus, the shape of the curves between these values apparently reflected subtle changes in the size and light-scattering properties of the microcrystals themselves (a conclusion supported by EM observations).

The pH dependence of microcrystal formation was examined for several different anion systems. In both the acetate and chloride systems, microcrystal formation occurred uniformly between pH 7.0 and 8.5 (data not shown). Polymin-P was found to have a strongly inhibitory effect on crystallization even at 0.1% concentrations.

The maximum turbidity of the RecA protein solutions after addition of spermidine–acetate (pH 7.5) increased as a linear function of protein concentration. When spermidine acetate (pH 7.5) was added to 7 mM to 20 mM Tris–acetate (pH 7.5) solutions containing from 10 to 800 μg/mL RecA protein, the turbidity showed a linear increase from 0.05 to 1.8 OD_{320} units (Figure 5). When the spermidine concentration was decreased 10-fold, the increase in turbidity remained linear but at a

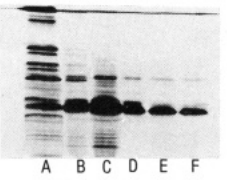

FIGURE 6: Purification of RecA protein utilizing a microcrystallization step. RecA protein was purified from 65 g of cells as described under Experimental Procedures. Aliquots from fractions 1 (A), 2 (B), 3 (C), 4 (D), 5 (E), and 6 (F) were electrophoresed on a 7.5% SDS–polyacrylamide gel and stained with a silver stain. The purification produced by spermidine microcrystallization is seen by comparing lanes C and D.

reduced level (Figure 5). This increase was dependent only on the presence of RecA protein; including 1 mg/mL bovine serum albumin in each sample did not affect the results even at very low RecA protein concentrations (Figure 5).

Because spermidine itself might alter RecA protein dependent reactions were it present in appreciable amounts, the binding of spermidine to RecA protein in the microcrystals was examined by using ^{14}C-labeled spermidine (Amersham). The labeled spermidine was diluted with cold 0.5 M spermidine acetate (pH 7.5) and the mixture used to induce microcrystal formation of 100 μg of previously purified RecA protein. When the protein microcrystals were collected by centrifugation and counted, it was found that there was less than 1 molecule of spermidine in the microcrystals for every 10 RecA protein monomers. Brief dialysis against buffer A reduced the level of ^{14}C label to undetectable levels. When 100 μg of RecA protein in 0.01 M Tris–acetate (pH 7.5) and 10 mM $MgCl_2$ was incubated with 25 μg of DNase I or RNase for 30 min at 37 °C and then mixed with 7 mM spermidine, full microcrystallization was observed, indicating that microcrystal formation is not nucleated by contaminating nucleic acids.

Purification of RecA Protein by Spermidine Microcrystallization. Spermidine microcrystallization provides a valuable step in the purification of RecA protein. The purification described has been utilized routinely in this laboratory with excellent results and can be accomplished in 2–3 days time. In the preparation illustrated (Experimental Procedures), an extract from 65 g of cells was carried through steps of Polymin-P precipitation and ammonium sulfate extractions, yielding 2100 mg of protein constituting 10% RecA protein as judged by SDS–polyacrylamide gel analysis. The ATPase activity of this fraction (fraction 2) was stimulated 4-fold by addition of an excess of M13 ssDNA. Fraction 2 was then passed over a 2.5 × 30 cm phosphocellulose P-11 column. This step resulted in a 2-fold purification and removed traces of Polymin-P which inhibited crystallization. The resulting protein, 1100 mg, constituted 25% RecA protein, and its ATPase activity remained unchanged from that of fraction 2. Following dialysis, addition of spermidine to 7 mM led to the immediate formation of RecA protein microcrystals which were collected by centrifugation. This step yielded 240 mg of RecA protein with an OD_{280}/OD_{260} ratio of 1.67. As judged by SDS–polyacrylamide gels, the protein was greater than 90% pure (Figure 6), and the ATPase activity was stimulated 50-fold by the addition of an excess of M13 ssDNA (the activity in the absence of added ssDNA was only 2% of that measured in the presence of a saturating amount of added ssDNA). The RecA protein was very active in DNA binding as assayed by EM and in ssDNA-dependent LexA protein cleavage (not shown).

If further purification of the RecA protein was desired, gel filtration in high salt on Sephacryl S-200 (Kuramitsu et al., 1981) which should remove any remaining small molecular weight DNA or RNA bound to the RecA protein was used next, followed by chromatography on PBE-94 which has a very high capacity for RecA protein. In the preparation described here, no further purification was achieved by these two steps, as judged by its ATPase activity and appearance on gel electrophoresis. However, minor but active contaminants such as nucleases or polynucleotide phosphorylase may be further reduced by these steps. If poor crystal formation was observed following addition of spermidine, this was likely due to traces of Polymin-P remaining after the P-11 chromatography. In this case, brief dialysis against buffer A containing 7 mM spermidine yielded full crystallization.

Other schemes employing spermidine or spermine for the purification of RecA protein were examined but are not described here. Microcrystal formation was rapid and quantitative in crude lysates (fraction 1), but the microcrystals were contaminated by nucleic acids. Much of the nucleic acid could be removed by washing the microcrystals with solutions of 7 mM spermidine and 50 mM NaCl, which dissolved the RecA protein microcrystals but left the nucleic acid as a precipitate. This step followed by chromatography on PBE-94 appeared to provide a very rapid purification. Because some other proteins were associated with the microcrystals when they were formed in crude lysates (as observed by SDS gel electrophoresis), this procedure might provide a means of identifying proteins that interact with RecA protein in vivo.

Discussion

We have shown that RecA protein will rapidly form microcrystals in the presence of spermidine or spermine. Crystallization occurs rapidly in RecA protein solutions as dilute as 1 μg/mL or as concentrated as 10 mg/mL. Microcrystal formation was highly dependent on the counterions present, being most tolerant of acetate and strongly inhibited by sulfate. This unusual phenomenon provides a rapid and powerful purification step for the preparation of RecA protein from *E. coli* cell extracts. However, because spermidine or spermine may also precipitate DNA and RNA, it is important to include a step that removes nucleic acid.

We do not have an adequate explanation for this phenomenon and are not aware of other examples of spermidine-induced protein crystallization. We have observed nearly identical RecA protein microcrystals as judged by EM when RecA protein was concentrated to 100 mg/mL in the absence of any polyamines. It is possible that the polyamines somehow mimic the effects of high protein concentration. The increasing inhibition of crystal formation in the order of acetate < chloride < phosphate < sulfate may be due to a simple shielding of the charge on the polyamine. We found that spermidine does not remain tightly bound in the RecA protein microcrystals, eliminating the possiblity of stable polyamine cross-linking of the protein. Microcrystal formation also does not appear to be nucleated by nucleic acids.

As shown in Figure 1, the microcrystals appear by EM to be highly ordered, and they often appear as a bundle of long thin filaments, but this could be an artifact of the staining. Although the microcrystals are too small for single-crystal X-ray diffraction studies, EM image enhancement and analysis methods could aid in elucidating their structure. Possibly a more controlled exposure of RecA protein to spermidine or spermine might allow the growth of much larger crystals.

We have described a purification of RecA protein from *E. coli* cell extracts employing a crystallization step. We have routinely prepared 200 mg of RecA protein pure from 65 g of cells in 2–3 days. The RecA protein purified by this procedure is highly active in its formation of nucleoprotein fila-

ments with DNA as seen by EM and has ATPase and LexA repressor cleavage activities that are highly ssDNA dependent. RecA protein purified by this procedure does not form appreciable amounts of filaments when incubated with ATPγS and Mg^{2+}, a property we will show elsewhere is due to contaminating RNA and polynucleotide phosphorylase. There are other stages in the purification of RecA protein at which microcrystallization can be introduced, in particular very early. We have explored some of these schemes, and in time, it would be expected that even better purifications based on this phenomenon will be developed.

Registry No. ATPase, 9000-83-3; chloride, 16887-00-6; acetic acid, 64-19-7; sulfate, 14808-79-8; phosphate, 14265-44-2; spermidine, 124-20-9; spermine, 71-44-3; polymin P, 74913-72-7.

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Determination of the Relative Positions of Amino Acids by Partial Specific Cleavages of End-Labeled Proteins†

Rodney A. Jue‡ and Russell F. Doolittle*

Department of Chemistry, University of California, San Diego, La Jolla, California 92093

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ABSTRACT: We have developed a new method for obtaining information about protein sequences that uses an approach analogous to that used to determine DNA sequences. In essence, three steps are involved. First, a detectable label is attached exclusively to the amino terminus of a polypeptide. Next, the labeled chain is subjected to partial specific cleavage in a way that produces roughly equimolar amounts of fragments of different sizes. Cleavages for methionine, tryptophan, arginine, aspartyl-proline bonds, and asparaginyl-glycine bonds have been employed. Lastly, the labeled fragments are separated according to size by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The distribution of target amino acids along the polypeptide chain can be deduced from the specific pattern of labeled bands by reading the "ladder" in the same way that DNA sequencing gels are read. Although the method can be conducted with a radioactive label, we have chosen to use a fluorescent label. We have applied the method successfully to the three subunit chains of two different fibrinogens.

Tremendous advances in genetics and biochemistry have been made possible by recent progress in DNA sequencing that depends on polyacrylamide gel electrophoresis. Many researchers use the biosynthetic incorporation of radioactive nucleotides, as introduced by Sanger et al. (1977), to generate a "ladder" that allows the direct reading of DNA sequences from polyacrylamide gels. Alternatively, the same result is achieved by the Maxam & Gilbert (1977) approach in which DNA that is exclusively labeled at one end is randomly fragmented at locations occupied by only one of the four bases. The development of an analogous sequencing procedure applicable to proteins must surmount a number of obstacles. In the first place, neither protein terminus easily lends itself to specific labeling, because the terminal amino group is similar

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‡ Present address: Hybritech, Inc., 9850 Distribution Avenue, San Diego, CA 92121.

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