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The *recBC* Deoxyribonuclease of *Escherichia coli*: Isolation and Characterization of the Subunit Proteins and Reconstitution of the Enzyme

(recombination/complementation/ATP)

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ABSTRACT **After dissociation of the *E. coli recBC* DNase (ATP-dependent DNase) with concentrated NaCl, two subunit proteins were isolated by ion exchange chromatography. Combination and subsequent incubation of the subunits resulted in the appearance of the original DNase. The subunit proteins, designated α and β, have $s_{20,w}$ of 4.1 S and 8.1 S, respectively. The α subunit possesses neither the ATP-dependent DNase nor the DNA-dependent ATPase of the original enzyme. The β subunit contains a low level of both enzymatic activities in a ratio markedly different from that of the original enzyme. The β subunit complemented extracts from both *recB* and *recC* mutant strains to produce *recBC* DNase, while the α subunit did not complement either extract. These results suggest that *recB* and *recC* genes are both required for the production of β subunit and that the *recBC* DNase molecule contains a protein component (α) that is not determined by either the *recB* or the *recC* gene.**

The genetic recombination proficiency of *Escherichia coli* appears to depend on the functioning of several genes (1). Mutations in two of these, *recB* and *recC*, result in identical phenotypes with regard to reduced recombination efficiency and increased sensitivity to ultraviolet light and x-ray (2–4). Both *recB* and *recC* genes are known to affect the production of an ATP-dependent DNase, the *recBC* DNase, for the enzyme activity is not detected in extracts of either $recB^-$ or $recC^-$ mutant strains (5–7). Recently, we reported the formation of ATP-dependent DNase in the mixture of crude extracts from $recB^-$ and $recC^-$ mutant strains (8). These experiments suggested that the procedures used for the complementation *in vitro* might be applied to the isolation of possible protein subunits from the purified enzyme. Isolation and characterization of subunits of the *recBC* DNase would be an important step in fully understanding the complicated reactions catalyzed by the enzyme and, therefore, in revealing the exact role of the enzyme in genetic recombination and DNA repair. In this paper, we report the isolation of such subunit proteins, reconstitution of the enzyme molecule, and some biochemical and genetic properties of the subunits.

MATERIALS AND METHODS

Strains. E. coli (F^-) strains used in this study are given in Table 1.

Preparation of recBC DNase. The enzyme used here was prepared by the method of Nobrega *et al.* (9), with omission of DEAE–cellulose chromatography.

Abbreviations: Buffer A, 20 mM Tris·HCl (pH 7.8), 2 M glycerol, 0.1 mM EDTA, 50 mM 2-mercaptoethanol.

Enzyme and Protein Assays. The *recBC* DNase and ATPase were assayed as described (9, 10), except that T4 [^{3}H]DNA was used as substrate. One unit of DNase is defined as the amount catalyzing the conversion of 1 nmol of DNA nucleotide equivalent to an acid-soluble form in 20 min at 30°. Protein concentration was determined by the method of Lowry *et al.* (11).

Reconstitution of the Enzyme. The enzyme was reconstituted from subunit proteins (complementation of subunits) by incubating the mixture of subunits in buffer B [20 mM Tris·HCl (pH 7.8), 1 mM EDTA, 50 mM 2-mercaptoethanol, 0.15 M NaCl, 2 M glycerol, and 1 mg/ml of bovine serum albumin] for 2–4 hr at 23°. Complementation activity of subunit preparations was determined by incubating one subunit preparation with saturating amounts of the other subunit preparation. One unit of complementation activity is the amount of the subunits that produces 1 unit of DNase under the conditions described above.

RESULTS AND DISCUSSION

Isolation of Subunit Proteins. We reported previously that the *recBC* DNase activity was rapidly lost when the purified enzyme preparation was exposed to a high salt concentration (8). In the presence of 4 M NaCl (0°, pH 8.0), the enzyme lost 98% of its activity within 30 min. However, when the

TABLE 1. *E. coli strains used in this study*

Strain	Rec mutation	Other markers	Source
AB1157	*recB*$^+$ *recC*$^+$	*thi-1, thr-1, leu-6, lacY1, mtl-1, xyl-5, ara-14, galK2, his-4, proA2, argE3, tsx-33, sup-37* (amber), *str-31*	K. B. Low
AB2470	*recB21*	Same as AB1157	K. B. Low
NH4033	*recC22*	Same as AB1157 except *his*$^+$	K. B. Low
JC5519	*recB21 recC22*	Same as AB1157	A. J. Clark
JC5723	*recB95*	Same as AB1157, except *leu-1, supE44* (does not bear *leu-6, sup-37*)	A. J. Clark
JC4704	*recC155*	Same as JC5723 except *thyA*$^-$, *su*$^-$	A. J. Clark
JC4705	*recB156*	Same as JC5723 except *thyA*$^-$, *su*$^-$	A. J. Clark
KL254	*recA1 recB21*	Same as AB1157 except *nalA*^{r}	K. B. Low

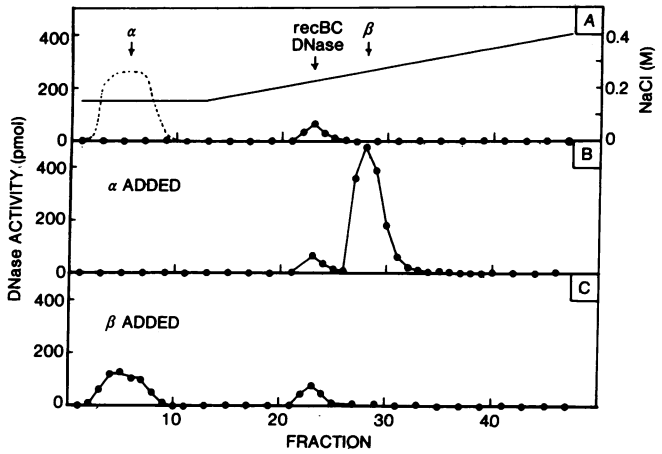

FIG. 1. Separation of DNase into two subunit fractions. To a purified DNase preparation (fraction VI, 141 units) in 750 μl of buffer A [20 mM Tris·HCl (pH 7.8), 2 M glycerol, 0.1 mM EDTA, 50 mM 2-mercaptoethanol], 174 mg of solid NaCl was added (final NaCl concentration, 3.7 M). The preparation was held at 0° for 2 hr, then dialyzed against buffer A containing 0.15 M NaCl for 1 hr at 0°. The NaCl concentration was then adjusted to 0.15 M by addition of buffer A (final volume, 3.5 ml). After the sample was mixed with a marker (human hemoglobin, 0.42 A_{540} unit in 5 μl), it was applied to a column of DEAE-Sephadex A-25 (0.9 × 4.5 cm) that had been equilibrated with buffer B. The column was washed with 6 ml of buffer B and elution was carried out with a continuous linear gradient of 0.15–0.45 M NaCl in buffer B. The entire column effluent was collected in 0.77-ml fractions to which 75 μg of bovine serum albumin (in 5 μl) had been previously added. (A) Aliquots (10 μl) of each fraction were assayed for DNase activity (●) and NaCl (solid curve). Hemoglobin (A_{540}) (broken curve). (B) Aliquots (0.5 ml) of fractions 3–7 were pooled (α fraction) and 40 μl of α fraction, 50 μl of buffer A, 1 μl of 100 mg/ml of bovine serum albumin, and 10 μl each from column fraction were mixed and incubated for 5 hr at 23°. Each mixture was then assayed for DNase activity (●). (C) Aliquots (0.34 ml each) of fractions 27–29 were pooled (β fraction), and 30 μl of β fraction, 60 μl of buffer A, 1 μl of 100 mg/ml of bovine serum albumin, and 10 μl from each column fraction were mixed and incubated for 5 hr at 23°. Each mixture was then assayed for DNase activity (●).

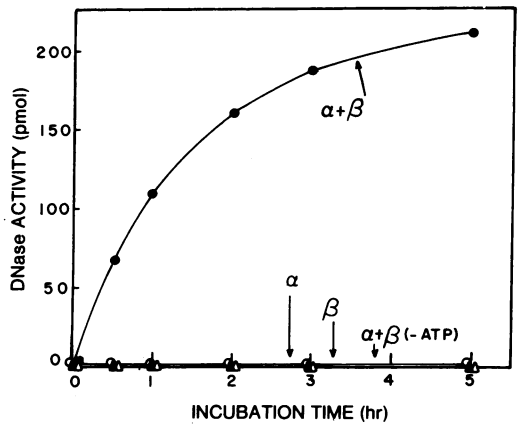

FIG. 2. Reconstitution of *recBC* DNase as a function of incubation time. Subunits α (450 μl) and β (300 μl), prepared as described in the legend of Fig. 1, were mixed and incubated at 23° in buffer B. At various time intervals, 50-μl aliquots were withdrawn and assayed for DNase activity in the presence (●) and absence (○) of ATP. For controls, subunits α and β were incubated separately; aliquots of subunits α (30 μl) and β (20 μl) were withdrawn and assayed for DNase activity in the presence of ATP: α subunit, ▲; β subunit, △.

salt was removed from the preparation, up to 100% of the original enzyme activity was gradually restored. These and other experimental results suggested that the inactivation and reactivation processes could be best explained as the dissociation and reassociation of enzyme subunits. Based on these findings, we attempted to isolate possible subunit proteins from the salt-denatured DNase and to reconstitute the original enzyme.

The purified enzyme preparation was first inactivated with 3.7 M NaCl, and the salt concentration was reduced to 0.15 M by dialysis and dilution. The sample was immediately applied to a column of DEAE-Sephadex. After the fractions that were not absorbed to the column at this salt concentration (0.15 M) were collected, linear NaCl gradient chromatography (0.15–0.45 M) was performed. As shown in Fig. 1A, a small amount of DNase activity was detected at 0.23 M NaCl, at which concentration the *recBC* DNase is normally eluted. This activity presumably resulted from reconstitution of the enzyme during manipulation before the chromatography or from enzyme that escaped salt denaturation. However, when the material not absorbed to the column at 0.15 M NaCl was combined with each of the gradient chromatography fractions, a DNase activity emerged with the fractions eluted between 0.26 M and 0.28 M NaCl (Fig. 1B). The same results were obtained by a reciprocal reconstitution experiment, in which the fractions eluted between 0.26 and 0.28 M NaCl were pooled and combined with each of the other fractions. As seen in Fig. 1C, the only fractions that produced active enzyme when combined with the 0.26–0.28 M fractions were the unadsorbed fractions. At least 25% of the original enzyme activity was recovered from the subunit preparations by complementation. The DNase that emerged exhibited an absolute ATP requirement for the enzyme activity. It also had the same sedimentation rate ($s_{20,w}$, 12.4 S) and heat stability (half-life 30 min at 47.5°) as the original enzyme (data not shown). These results suggested that the DNase molecules were dissociated into two subunits by the NaCl treatment and that active enzyme molecules were reconstituted when these subunits were recombined.

The reconstitution of the enzyme molecule from subunits is a time-dependent process (Fig. 2). Under these conditions, reconstitution was nearly complete within 2 hr. Incubation of either fraction alone did not produce a rise in enzyme activity. The reconstitution process was also affected by subunit concentration, incubation temperature (optimum 23°), and salt concentration (optimum 0.10–0.15 M NaCl) but unaffected by the presence of DNA and ATP. Detailed studies of conditions affecting the reconstitution process will be reported elsewhere. We have designated the active material in the 0.15 M salt eluate as α fraction (subunit) and the one in the 0.26–0.28 M eluate as β fraction (subunit). The complementation activities of the subunit preparations were determined by incubating one subunit with an excess of the other subunit.

Characterization of the Subunit Proteins. The active components in the α and β fractions were nondialyzable and lost more than 99% of their reconstituting activities after incubation at 65° for 15 min, indicating the protein nature of

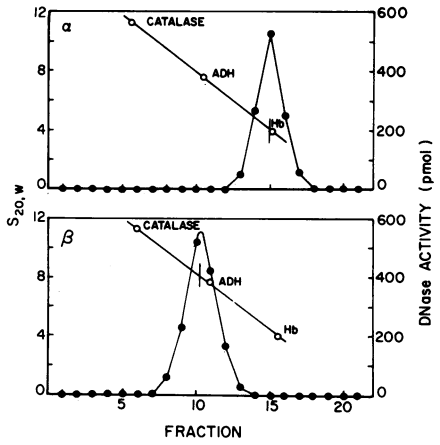

FIG. 3. Sedimentation of the subunits. Twenty units of subunit α or β were mixed with catalase (Worthington CTR, 2000 units, 0.08 mg), yeast alcohol dehydrogenase (ADH, Worthington ADHS, 0.31 unit, 0.039 mg), and hemoglobin (0.84 A_{540} unit) in 1 ml of buffer C [20 mM Tris·HCl (pH 7.6), 0.4 M glycerol, 1 mM EDTA, 50 mM 2-mercaptoethanol, 0.2 M NaCl] and layered on a 10.8-ml gradient of 1.5–3 M glycerol in buffer C containing 0.1 mg/ml of bovine serum albumin. Gradients were centrifuged at 37,000 rpm for 32 hr at 2° in a Beckman SW 41 rotor. Under these conditions, the $s_{20,w}$ of hemoglobin is 4.0 S. Hemoglobin was assayed by A_{540}; catalase by the decrease of A_{240} when 5 μl of a gradient fraction was added to 1 ml of substrate (0.06% H_2O_2, 50 mM phosphate buffer, pH 7.0); and alcohol dehydrogenase by the increase of A_{340} when 10 μl of a gradient fraction was added to 0.3 ml of substrate [16 mM Tris·HCl (pH 8.8), 8 mM NAD, 0.33 M ethanol]. Subunit α was assayed by combining 50 μl of each gradient fraction with 50 μl (0.5 unit) of β subunit in buffer A containing 1 mg/ml of bovine serum albumin, incubating at 23° for 9 hr, and assaying for DNase activity. Subunit β was assayed in the same way, with 1.2 units of α subunit per assay.

the subunits. The sedimentation pattern of each subunit was examined by centrifuging each subunit with reference markers in glycerol gradients. As shown in Fig. 3, each subunit preparation showed a single symmetrical complementation activity, and therefore seems to contain a single class of molecules. The $s_{20,w}$ values of the subunits were calculated to be 4.1 S for α and 8.1 S for β. If we assume their shapes to be globular, the α and β subunits should have molecular weights of about 60,000 and 170,000, respectively. (Because of the limited quantities of pure subunit preparations currently available, we have not yet determined the molecular weight of these subunit proteins by other means.) Goldmark and Linn observed two protein bands corresponding to polypeptides of molecular weights 128,000 and 140,000 in sodium dodecyl sulfate gel electrophoresis of the purified enzyme (12). The molecular weight of the intact enzyme was estimated to be 350,000 from sedimentation analysis (12.4 S) (6). However, these results are still insufficient to determine precise subunit composition of the enzyme molecule.

The *recBC* DNase had been found to possess not only an ATP-dependent DNase, but a DNA-dependent ATPase as well (6, 9, 12). We tested for both these enzyme activities in α and β subunit preparations. As shown in Table 2, we were unable to detect any of these enzyme activities in the α subunit. On the other hand, β subunit retained a small but significant ATP-dependent DNase and DNA-dependent ATPase activity. The DNase activity in β subunit alone was

TABLE 2. *Enzymatic activities associated with subunits and native enzyme*

Enzymatic activities (substrate or cofactor)	α Subunit	β Subunit	Native enzyme
Complementation activity as DNase	5.02	5.96	—
DNase (dsDNA)	<0.001	0.023	0.720
DNase (ssDNA)	<0.001	0.002	0.022
DNase (dsDNA, no ATP)	—	<0.001	<0.001
Complementation activity as ATPase	30.1	35.8	—
ATPase (dsDNA)	<0.06	2.37	4.36
ATPase (ssDNA)	<0.06	0.21	0.28
ATPase (no DNA)	—	<0.06	<0.06
DNase (dsDNA)/complementation activity as DNase	—	0.0038	—
ATPase (dsDNA)/complementation activity as ATPase	—	0.067	—
ATPase (dsDNA)/DNase (dsDNA)	—	103	6

Before dissociation, *recBC* DNase (fraction VI, 480 units) was further purified by ultracentrifugation as described in the legend of Fig. 3, with the gradient containing 0.05 M NaCl. Subunits were then prepared as described in the legend of Fig. 1, except that hemoglobin was omitted. The β subunit was further purified by ultracentrifugation with the gradient containing 1 mg/ml of bovine serum albumin and 0.15 M NaCl as described in the legend of Fig. 3. Subunit complementation activities (as DNase) were determined by incubating 1 subunit with an excess of the other subunit. Complementation activities as ATPase were calculated from complementation activities as DNase by multiplication by 6.0. All enzyme activities shown above are expressed in pmol hydrolyzed (ATP) or made acid-soluble (DNA nucleotide equivalent) under standard conditions. All assays were performed in duplicate. ds, double-stranded; ss, single-stranded. —, not done.

about 0.4% of the activity it exhibited when complemented by excess α subunit, and the ATPase activity was 7% of the potential (complementation) ATPase activity. This residual DNase and ATPase activity found in the β subunit preparation was not due to contamination by a trace of the original *recBC* DNase, because these activities sedimented at the same rate (8.1 S) as the complementation activity of β subunit in glycerol gradient centrifugation (data not shown). The DNase and ATPase activities in the β subunit preparation were similar to those observed with the *recBC* DNase in such basic characteristics as their preference for double-stranded DNA (dsDNA) over single-stranded DNA (ssDNA) substrates (Table 2). However, the ratio of ATP to DNA hydrolysis (ATPase/DNase) by β subunit alone is extremely high (about 100 ATP molecules hydrolyzed per DNA nucleotide made acid-soluble) compared to the native or reconstituted enzyme, in which the ratio is about 6. The results presented here suggest that β subunit contains catalytic sites for each DNase and ATPase reaction and is the core protein of the *recBC* DNase; and that α protein, by binding to β subunit, enables the β subunit to function in a more efficient manner such that fewer ATP molecules are consumed during hydrolysis of a single DNA phosphodiester bond. It is also possible that the α subunit induces qualitative changes in reactions executed by the β subunit.

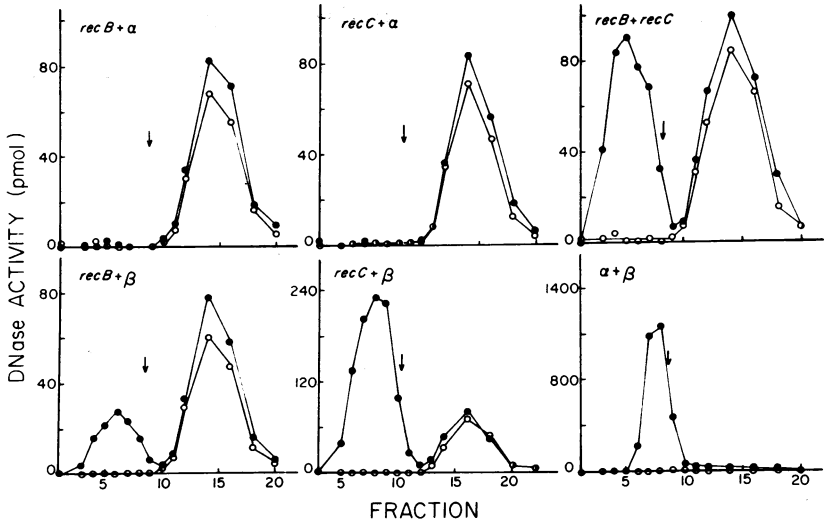

FIG. 4. Complementation between subunits and extracts of $recB^-$ and $recC^-$ mutants. Subunits were prepared as described in the legend of Fig. 1. The β subunit was further purified by glycerol gradient centrifugation as described in the legend of Fig. 3. Strains harboring *recB21* (AB2470) and *recC22* (NH4033) mutations were grown to late logarithmic phase in G medium (1 liter) (9) and quickly chilled. Cells were washed twice in buffer D [0.1 M Tris·HCl (pH 7.6), 1 M glycerol, 1 mM EDTA, 50 mM 2-mercaptoethanol, 0.3 M NaCl], resuspended in 4 ml of the same buffer, and disrupted by Ratheon Sonic Oscillator at maximum power (three 1-min bursts). Cell debris was removed by centrifugation (18,000 rpm, 30 min, 0°) and the protein concentration of the supernatant was adjusted to 17.4 mg/ml by addition of buffer D. Then 3 ml of each extract was added to 0.3 ml of 20% (w/w) Dextran T500 (Pharmacia), 0.9 ml of 30% (w/w) polyethylene glycol, and 984 mg of NaCl. After the NaCl was dissolved by gentle mixing, the mixtures were centrifuged (5000 rpm, 10 min, 0°) and the upper phase was dialyzed overnight at 0° against 1 liter of buffer A containing 0.3 M NaCl. Subunits α or β (15 units in 0.1 ml of buffer A containing 1 mg/ml of bovine serum albumin and 0.3 M NaCl) were then combined with $recB^-$ or $recC^-$ extracts, each combination containing 0.1 ml of subunit, 0.1 ml of extract, and 0.2 ml of buffer A. Each mixture was incubated at 23° for 4 hr, diluted with 0.8 ml of buffer [20 mM Tris·HCl (pH 7.6), 0.1 M NaCl], mixed with 1000 units of catalase, and layered on a 10.4-ml gradient of 1.5–3 M glycerol in buffer C containing 0.1 M NaCl. After centrifugation (SW 41 rotor, 37,000 rpm, 23 hr, 2°) 0.15 ml of each fraction was assayed for DNase activity in the presence (●) and absence (○) of ATP. Arrows indicate the position of the catalase marker.

Genetic Origin of α and β Subunits. One interesting aspect of the subunit proteins is their genetic origin. We attempted to identify the genetic origin of the two subunit proteins by complementation between purified subunit preparations and extracts from *recB* and *recC* mutant strains. A DNA-free extract from either strain AB2470 (*recB21*, missense or frameshift mutation) or NH4033 (*recC22*, opal nonsense mutation) was incubated with α or β subunit and the samples were fractionated by glycerol gradient centrifugation. The *recBC* DNase activity produced by complementation could be distinguished from other DNase activities present in the extracts by its ATP dependency and its faster sedimentation rate (12.4 S). Fig. 4 shows the results of these experiments. Unexpectedly, we found that α subunit did not produce any ATP-dependent DNase upon incubation with either *recB* or *recC* extracts, while β subunit produced such a DNase with either *recB* or *recC* extract. An ATP-dependent DNase was also produced upon incubation of β subunit with extracts of JC5519, a *recB21* and *recC22* double mutant strain. The characteristics of the DNase produced by this complementation were identical to the *recBC* DNase in (*a*) chromatographic behavior on DEAE-Sephadex (elution at 0.23 M NaCl), (*b*) sedimentation rate in glycerol gradients (12.4 S), and (*c*) heat stability (Fig. 5). Also, the active material in the extract from a $recB^- recC^-$ mutant strain behaved on DEAE-Sephadex chromatography in the same manner as the α subunit obtained from a purified enzyme preparation. These results suggest that α subunit is present in and β subunit is absent from both $recB^-$ and $recC^-$ strains. Therefore, the synthesis of the α subunit appears to be independent of these genes, while the synthesis of β subunit requires both genes. In order to confirm the above findings, we have performed similar complementation experiments using other *recB* and *recC* mutant strains (kindly provided by Dr. A. J. Clark). As shown in Table 3, extracts of JC5723 (*recB95*, opal nonsense mutation), JC4705 (*recB156*, amber nonsense mutation), and JC4704 (*recC155*, amber nonsense mutation) all produced ATP-dependent DNase upon incubation with β subunit, although the amount of activity varied with the mutant strain used. This confirmed the presence of α subunit in $recB^-$ and $recC^-$ mutant strains.

These results provide some insight into the genetic origins of the subunits. From the results presented here and the fact that a *recB* temperature-sensitive (*ts*) mutant produces temperature-sensitive *recBC* DNase (13), it seems quite clear that the *recB* gene codes for at least part of the β subunit. On the other hand, because the only available *recC ts* mutant strain does not show temperature-sensitive DNase activity (13), two possibilities should be considered for the role of the *recC* gene in β subunit synthesis. The *recC* gene product is either a structural component of the β subunit or an enzyme that modifies the *recB* gene product (β subunit precursor) to form active β subunit. The possibility that the *recC* gene regulates *recB* gene product synthesis has been excluded by showing *in vitro* complementation between *recB* and *recC* mutant extracts (8).

The genetic origin of the α subunit is not known. The other major recombination gene, *recA*, has been ruled out for sev-

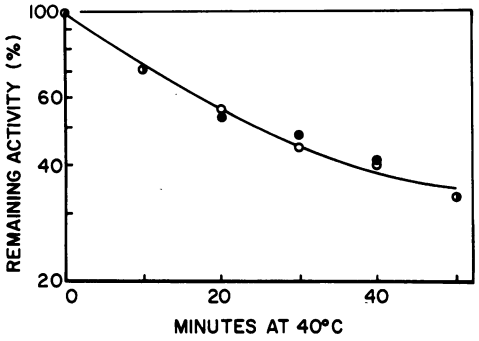

FIG. 5. Heat inactivation of the *recBC* DNase. Subunit β and an extract from JC5519 (*recB21 recC22*) were prepared as described in the legend of Fig. 4. To 6.5 ml of dialyzed extract from the mutant strain, 50 μl of hemoglobin solution (4.2 A_{540} units) and 6.9 ml of buffer A were added and the mixture was passed through a column of DEAE-Sephadex A25 (0.9 × 7.9 cm) equilibrated with buffer B. The column was washed with 10 ml of buffer B. Eluate containing unadsorbed protein was pooled (12.8 ml), combined with 134 units of subunit β in 0.61 ml of buffer B, incubated for 4 hr at 23°, and passed through a second DEAE-Sephadex column (0.9 × 4.7 cm) equilibrated with buffer B. The column was washed with 10 ml of buffer B and eluted with a gradient of 0.15–0.45 M NaCl in a total of 60 ml of buffer B. The eluate was collected in 1-ml fractions to which 0.1 mg of bovine serum albumin was added. Fractions having DNase activities were pooled (1.9 ml) and dialyzed twice against 1 liter of buffer B. Native *recBC* DNase (31 units in 7.5 ml of buffer B containing 0.1 mg/ml of bovine serum albumin) was also dialyzed in the same way. Each enzyme preparation was then diluted with an equal volume of 100 mg/ml of bovine serum albumin. Aliquots (50 μl) were exposed to 40° for varying periods, shown in the figure, and assayed for DNase activity. ●, Complementation product; ○, native *recBC* DNase.

eral reasons. Various *recA* mutants possess at least normal levels of *recBC* DNase, and *in vitro* complementation of an extract of a *recA recB* double mutant with β subunit did yield the *recBC* DNase activity (Table 3). The failure to date to find *rec*$^-$ mutants (besides *recB* and *recC* strains) that affect the level of *recBC* DNase suggests several possibilities. The least likely is that among the many *rec*$^-$ strains isolated to date, by chance none has had a mutation in the gene coding for an α subunit protein that is essential for recombination. Alternatively, α protein may have roles both in recombination and in some essential cellular function, such as DNA replication, catalyzing a reaction common to both functions. A protein of this kind has already been found in bacteriophage T4 (14). A mutation in a gene for such a protein, unless conditional, would be lethal, and, therefore, the mutants could not be isolated by the procedures generally used to obtain *rec*$^-$ mutant strains. Another possibility is that α plays little or no part in recombination. The β subunit alone may be the primary recombination enzyme, while the $\alpha\beta$ complex may have an additional function. In this case, strains bearing mutations in the gene for α would not be recombination-deficient (or only slightly deficient). It is important in this regard to see whether qualitative differences exist between the DNase reactions catalyzed by β subunit and the intact *recBC* DNase.

TABLE 3. *Complementation of subunits with extracts of rec*$^-$ *strains*

	DNase activity (pmol)
(A) AB2470(*recB21*) + α	<5
NH4033(*recC22*) + α	<5
AB2470(*recB21*) + β	404
NH4033(*recC22*) + β	3560
AB2470(*recB21*) + NH4033(*recC22*)	1470
α + β	11700
(B) JC5723(*recB95*) alone	51
JC5723(*recB95*) + β	2840
JC4705(*recB156*) alone	<5
JC4705(*recB156*) + β	500
JC4704(*recC155*) alone	<5
JC4704(*recC155*) + β	2390
JC5519(*recB21recC22*) alone	<5
JC5519(*recB21recC22*) + β	269
KL254(*recA1recB21*) alone	<5
KL254(*recA1recB21*) + β	137
β alone	<5

(A) *recBC* DNase activity was calculated by summing the activity in fractions 3–9 of each gradient in Fig. 4. (B) Extracts from strains JC5519 (*recB21 recC22*), JC4723 (*recB95*), KL254 (*recA1 recB21*), and JC4704 (*recC155*) were incubated with or without β subunit, and centrifuged as described in the legend of Fig. 4. *recBC* DNase activity was calculated by summing the activity in fractions 3–9 of each gradient.

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