Transformation in Bacillus subtilis: Further Characterization of a 75,000-Dalton Protein Complex Involved in Binding and Entry of Donor DNA

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A 75,000-dalton protein complex purified from membranes of competent Bacillus subtilis cells was previously shown to be involved in both binding and entry of donor DNA during transformation. The complex, consisting of two polypeptides, a and b, in approximately equal amounts, showed strong DNA binding as well as nuclease activity (H. Smith, K. Wiersma, S. Bron, and G. Venema, J. Bacteriol. 156:101–108, 1983). In the present experiments, peptide mapping indicated that the two polypeptides are not related. Chromatography on benzoylated, naphthoylated DEAE-cellulose showed that polypeptide b generated single-stranded regions in double-stranded DNA. A considerable amount of the DNA was rendered acid soluble by polypeptide b. The nuclease activity of polypeptide b was reduced in the presence of polypeptide a. This resulted in an increased fraction of high-molecular-weight double-stranded DNA containing single-stranded regions. The acid-soluble DNA degradation products formed by polypeptide b consisted exclusively of oligonucleotides. In contrast to its nuclease activity, which was specifically directed toward double-stranded DNA, the DNA binding of the native 75,000-dalton complex to single-stranded DNA was at least as efficient as to double-stranded DNA.

Recently, several studies on binding and entry of donor DNA during bacterial transformation have rapidly increased our understanding of these processes. A successful approach has been the isolation and characterization of mutants deficient in either DNA binding or DNA entry (13, 14, 20–22). At least two proteins are likely to be involved in the early steps of transformation of Streptococcus pneumoniae. A role of a membrane-located protein in DNA binding has been suggested (7, 19). The major endonuclease of S. pneumoniae, which is also membrane located, was found to be required for DNA entry (9). Single-stranded nicks are introduced in donor DNA on binding to S. pneumoniae cells (8). Evidence has been presented that the major endonuclease introduces breaks opposite the initial nicks. It has been suggested (7) that the resulting double-stranded cleavage initiates DNA entry. During entry, one strand of the donor DNA is degraded by the major endonuclease, whereas the complementary strand enters the cell.

Recently, we presented studies on the mechanism of DNA binding and entry during transformation in Bacillus subtilis. By isolating and characterizing mutants deficient in the initial stages of transformation, we have established that a 75,000-dalton protein complex is involved in both binding and entry. This protein complex, purified from membranes of competent B. subtilis cells, consisted of two polypeptides, a (molecular weight, 18,000; isoelectric point, 5.0) and b (molecular weight, 17,000; isoelectric point, 4.7), in approximately equal amounts. Mutants which lacked polypeptide a (but not b) were DNA binding deficient (20), whereas mutants which lacked polypeptide b were DNA entry deficient (14, 22). The purified 75,000-dalton protein complex showed strong DNA binding as well as nuclease activity. The nuclease activity was dependent on the presence of divalent cations and was directed specifically toward double-stranded DNA (21). Analysis of the separated polypeptides a and b revealed that for effective DNA binding, the interaction of both polypeptides in the native 75,000-dalton complex was required. The nuclease activity was restricted to polypeptide b (21). Analysis of the nuclease subunit b on DNA-containing polyacrylamide gels revealed nuclease activity at positions identical to those of the major competence-specific nuclease activities which were previously implicated in the entry of donor DNA during transformation (14, 22). These results indicated that in B. subtilis the polypeptides involved in DNA binding and entry are associated into a multifunctional protein complex.

The purpose of the present experiments was to increase our understanding of the role of polypeptides a and b and the 75,000-dalton complex in transformation by correlating in vitro properties of these proteins with known phenomena associated with the early steps in transformation of B. subtilis.

MATERIALS AND METHODS

Strains. The strains used were all derivatives of B. subtilis 8G-5 (1). Stocks of bacteriophage SPP1 were from our laboratory collection.

Media. Minimal medium and starvation medium used in the competence regimen were as described previously (1). Restriction endonucleases HaeIII and EcoRI were obtained from Boehringer GmbH, Mannheim, Federal Republic of Germany, and were used as recommended by the manufacturer. Standard agarose gel electrophoresis buffer contained 90 mM Tris borate (pH 8.3) and 2.3 mM EDTA. Unless stated otherwise, all chemicals were obtained from BDH, Poole, England, or E. Merck AG, Darmstadt, Federal Republic of Germany.

DNAs. [methyl-3H]thymidine-labeled chromosomal B. subtilis DNA was obtained and purified as described previously (20). [methyl-3H]thymidine-labeled plasmid pC194 DNA was obtained from strain 2G-8 (trpC2, thy) grown in the presence of 1 mCi of [methyl-3H]thymidine (specific activity, 20.7 mCi/μg; Radiochemical Centre, Amersham, England) per 100 ml of minimal medium. The specific
activity of the DNA preparation obtained was $2 \times 10^5$ cpm/μg of DNA. Phage SPP1 was extracted from CsCl-purified stocks. SPP1 DNA fragments were labeled by nick translation with $[^{35}S]$dCTP (3,000 Ci/mmol; Radiochemical Centre), as indicated by the manufacturer, to a specific activity of approximately $5 \times 10^7$ cpm/μg of DNA. DNA fragments were extracted from agarose gels by electrophoresis onto NA-45 DEAE membranes (Schleicher & Schuell, Dassel, Federal Republic of Germany). Conditions for optimal binding and recovery of the DNA were as recommended by the manufacturer. DNAs were denatured by boiling for 10 min.

Preparation of competent cultures and transformation. The procedures used for the preparation of competent cultures and transformation were as described previously (20).

Purification of the 75,000-dalton protein complex and polypeptides a and b. The procedure used to purify the 75,000-dalton protein complex was as described previously (21). Samples containing the purified 75,000-dalton complex were separated into subunits on two-dimensional gels, and polypeptides a and b were extracted from these gels as described previously (22).

Polyacrylamide gel electrophoresis. Slab gel electrophoresis of proteins was done basically by the method of Laemmli (10), and two-dimensional gel electrophoresis was done by the method of O’Farrell (15). Details were as described previously (20). Proteins in the gels were stained with Coomassie brilliant blue or with silver, as described previously (21).

Peptide mapping of $^{35}$S-labeled polypeptides a and b. Cultures were grown to competence in minimal medium containing 4 μg of L-methionine per ml. During the last 2 h of the competence regimen, the L-methionine was replaced by 10 μCi of $[^{35}S]$methionine per ml (specific activity, 800 Ci/ mmol; New England Nuclear Corp., Boston, Mass.). Membrane vesicles were prepared from the labeled competent cells as described previously (20). Protein samples containing approximately $5 \times 10^6$ cpm of $^{35}$S were separated on two-dimensional gels as described previously (20). The positions of polypeptides a and b were identified by autoradiography of the wet gels on Kodak XAR-5 films, and polypeptides a and b were cut from the gel. Proper excision was verified by autoradiography of the remainder of the gel. Protein was extracted by grinding gel pieces in 100 μl of 25 mM Tris–92 mM glycerine buffer containing 0.1% sodium dodecyl sulfate. After the addition of bovine serum albumin to 200 μg/ml, the eluted proteins were precipitated with 20% cold trichloroacetic acid. After centrifugation, the pellets were dissolved in 30 μl of 125 mM Tris hydrochloride (pH 6.8)–0.5% sodium dodecyl sulfate and digested with 6 μl of α-chymotrypsin (5 mg/ml in 50% glycerol) for 105 min at 37°C by the method of Cleveland et al. (2). The degradation products were separated on 15% polyacrylamide slab gels and stained with Coomassie brilliant blue, and the labeled products were analyzed by fluorography.

Chromatography on BND-cellulose. $[^{3}H]$thymidine-labeled pC194 DNA was linearized at its unique HaelIII site. Samples (60 μl) containing 3 μg of DNA were incubated for 30 min at 37°C with polypeptides a and b in 20 mM Tris hydrochloride (pH 7.4)–10 mM β-mercaptoethanol–5% glycerol–10 mM MgCl₂. The reactions were terminated by the addition of 340 μl of 50 mM EDTA. Total and trichloroacetic acid-soluble radioactivity was determined in 200-μl portions of the reaction mixtures, as described previously (21). The remaining 200-μl portions of the reaction mixtures were applied to columns (0.8 by 2.0 cm) of benzoyleated, naphthoylated DEAE (BND)-cellulose (Bio-Rad Laboratories, Richmond, Calif.) equilibrated with 1 mM Tris hydrochloride (pH 8.0)–1 mM EDTA–0.3 M NaCl (0.3 M TEN buffer). After being washed with 5 ml of 0.3 M TEN buffer (flow rate, approximately 0.5 ml/min), DNA was eluted with 15 ml of TEN buffer containing 1.0 M NaCl, followed by a 80-ml gradient ranging from 0 to 10% caffeine in 1.0 M TEN buffer. The radioactivity in the fractions (1.2 ml) was determined by scintillation counting as described previously (20).

Analysis of acid-soluble degradation products. Samples (60 μl) containing 0.75 μg of $[^{3}H]$-labeled chromosomal B. subtilis DNA were incubated for 30 min at 37°C with polypeptides a and b in 20 mM Tris hydrochloride (pH 7.4)–10 mM β-mercaptoethanol–5% glycerol–10 mM MgCl₂. The reactions were stopped by the addition of 340 μl of 50 mM EDTA. Solubilized trichloroacetic acid was then added to 0.1 g/ml. After 30 min at 0°C, the samples were centrifuged for 30 min in an Eppendorf centrifuge. The supernatants were loaded onto Sephadex G-15 columns (0.6 by 75 cm). As reference markers thymidic acid (50 μg), thymidine (50 μg), thymine (50 μg), and blue dextran (500 μg) were added. The samples were eluted with 50 mM ammonium acetate (flow rate, approximately 12 ml/h). Fractions (1 ml) were analyzed for radioactivity and absorbance at 280 nm.

Assays of DNA binding and nuclease activity. Nuclease activity was assayed by measuring the liberation of acid-soluble products from radiolabeled DNA, as described previously (21). DNA binding was measured as the effect of the proteins on the mobility of DNA in polyacrylamide gels, as described previously (21).

RESULTS

Peptide mapping of the separated polypeptides a and b. To investigate the possibility that the complexed polypeptides a and b are related, for example, that b is a degradation product of polypeptide a, both polypeptides were isolated from two-dimensional gels and subjected to limited proteolytic digestion. The degradation products of polypeptides a and b were completely different (Fig. 1, lanes 3 and 4). The possibility that the different polypeptide
patterns resulted from different conditions during proteolysis was excluded by the observation that unlabeled bovine serum albumin, added as control protein, was digested to the same extent in the samples containing polypeptide a and polypeptide b (Fig. 1, lanes 1 and 2).

Analysis of macromolecular degradation products produced by polypeptide b. To investigate the possibility that the conversion of double- to single-stranded segments observed during DNA entry in transformation of *B. subtilis* (23) could be carried out by polypeptide b, *Hae*III-linearized 3H-labeled plasmid pC194 DNA was incubated with polypeptide b in the presence or absence of polypeptide a. Macromolecular DNA breakdown products were analyzed on BND-cellulose columns, on which completely double-stranded DNA can be separated from double-stranded DNA containing single-stranded regions (5). Incubation with polypeptide b alone resulted in the disappearance of almost all of the entirely double-stranded DNA (Fig. 2). In this case, a large fraction (41% of the input radioactivity) became acid soluble. The remainder eluted from the column at positions characteristic for double-stranded DNA containing single-stranded regions to various degrees. In contrast, in the presence of polypeptide a, only 6% of the input radioactivity was rendered acid soluble under the conditions described in Fig. 2. The reduced level of acid solubilization resulted in an increased fraction of high-molecular-weight double-stranded DNA containing single-stranded regions. The degree of single-strandedness of these molecules was on the average less than that of molecules obtained with polypeptide b alone. In a similar experiment, in which the amount of polypeptide a was doubled (results not shown), the DNA fraction containing partially single-stranded DNA was increased in comparison with the results presented in Fig. 2. These results indicated that polypeptide b was able to generate single-stranded regions in double-stranded DNA and that the level of this activity was reduced by polypeptide a.

Analysis of the acid-soluble DNA degradation products produced by polypeptide b. During entry into competent *B. subtilis* cells, acid-soluble donor DNA products are released into the medium (3, 6). These breakdown products consist of a minority of oligonucleotides and a majority of mononucleotides, nucleosides, and free bases. To study whether a similar pattern of acid-soluble degradation products was generated by polypeptide b, 3H-labeled chromosomal DNA was incubated with polypeptide b and the acid-soluble degradation products were analyzed by chromatography on Sephadex G-15. The results (Fig. 3) showed that all the acid-soluble degradation products eluted before the reference mononucleotide (thymidylic acid) and therefore consisted exclusively of oligonucleotides. Qualitatively similar results were obtained with polypeptide b in the presence of polypeptide a (results not shown).

**Effects of polypeptides a and b on single- and double-stranded DNA.** In *S. sanguis* and *S. pneumoniae* the entered

![Diagram](http://jb.asm.org/)
single-stranded donor DNA is complexed to a small, competence-specific protein (11, 17, 18), which renders the DNA resistant to digestion by several nucleases, including the pneumococcus endonuclease involved in DNA entry (12).

To analyze whether polypeptide a fulfills a comparable function, we investigated (i) the DNA-binding properties of polypeptide a and of the native 75,000-dalton complex to single-stranded DNA and (ii) the ability of polypeptide a to protect DNA against digestion by DNase I and micrococcal nuclease. The results demonstrated that the complex reduced the mobility of single-stranded DNA in polyacrylamide gels (Fig. 4, lane 2) at least as efficiently as double-stranded DNA (Fig. 4, lane 7), indicating similar binding activities for single- and double-stranded DNA. On the contrary, separated polypeptide a (Fig. 4, lane 3) and the mixture of polypeptides a and b (Fig. 4, lane 5) did not show detectable levels of DNA binding to single-stranded DNA. These results were confirmed by the observation that on sucrose gradients no cosedimentation of 35S-labeled polypeptide a and the mixture of labeled polypeptides a and b with 3H-labeled DNA could be detected (results not shown).

Table 1 shows the effect of polypeptide a on the nuclease activity of polypeptide b, DNase I, and micrococcal nuclease. The amount of acid-soluble products generated from double-stranded DNA by polypeptide b was considerably reduced (from 35.7 to 1.6%) in the presence of polypeptide a. As observed previously (21), polypeptide b had almost no effect on single-stranded DNA. In contrast to the inhibiting effect on polypeptide b, the activities of DNase I and micrococcal nuclease on both double- and single-stranded DNA were significantly stimulated by polypeptide a.

The effects of polypeptide a on various nucleases as described above were also demonstrated by analyzing the macromolecular products generated from double-stranded plasmid DNA. Plasmid pC194 DNA, which was extensively degraded by polypeptide b, was much less extensively degraded in the presence of polypeptide a (results not shown). On the contrary, the plasmid, which was only slightly nicked by DNase I, became extensively degraded in the presence of polypeptide a. Qualitatively similar, but to a lesser extent, was the effect of polypeptide a on micrococcal nuclease.

### DISCUSSION

The present results showed that polypeptides a and b of the 75,000-dalton protein complex in *B. subtilis* were distinct proteins. A role of the 75,000-dalton protein complex in DNA entry during transformation in *B. subtilis* was suggested by the present and previous observations. First, the absence of polypeptide a in DNA-binding-deficient mutants suggested the involvement of this subunit in DNA binding (20–22). In agreement with this idea, the present results showed a strong DNA-binding activity of the native 75,000-dalton protein complex for both double- and single-stranded DNA. The observation that neither polypeptide a alone nor the mixture of a and b showed DNA-binding activity toward double- or single-stranded DNA was surprising. A possible explanation is that the quantities of separated polypeptides that could be extracted from the gels were too low to be detected in the binding assays used. An alternative explanation is that polypeptide a has to associate with polypeptide b in the native 75,000-dalton complex to obtain the appropriate conformation for DNA binding. Second, the nuclease activity associated with polypeptide b was identical to the competence-specific nuclease activity involved in DNA entry in *B. subtilis* (14, 22). Further support for the involvement of polypeptide b in DNA entry was given by the present result that single-stranded regions were generated in double-stranded donor DNA in vitro. This strongly suggested that the entered single-stranded donor DNA fragments observed in transformation of *B. subtilis* (for a review see

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Amt of products (% of input radioactivity)</th>
</tr>
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<tbody>
<tr>
<td>Double-stranded DNA</td>
<td>Single-stranded DNA</td>
</tr>
<tr>
<td>Polypeptide b</td>
<td>35.7</td>
</tr>
<tr>
<td>Polypeptide b plus a</td>
<td>1.6</td>
</tr>
<tr>
<td>DNase I (1 μg/ml)</td>
<td>19.8</td>
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<tr>
<td>DNase I plus polypeptide a</td>
<td>72.8</td>
</tr>
<tr>
<td>Micrococcal nuclease (0.01 μg/ml)</td>
<td>5.0</td>
</tr>
<tr>
<td>Micrococcal nuclease plus polypeptide a</td>
<td>29.7</td>
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</tbody>
</table>

* Portions (2 μl) of the different nucleases were incubated for 30 min at 37°C with 1 μg of 3H-labeled *B. subtilis* DNA (10^6 cpm/μg of DNA) in 30 μl of 20 mM Tris hydrochloride (pH 7.4)–10 mM β-mercaptoethanol–5% glycerol–10 mM EDTA (pH 7.4)–10 mM MgCl₂ (10 mM CaCl₂ for micrococcal nuclease) in the presence or absence of two μl of polypeptide a. Reactions were terminated by the addition of 170 μl of buffer and 20 μl of cold trichloroacetic acid (100% wt/vol). After 30 min at 0°C, samples were centrifuged for 30 min in an Eppendorf centrifuge, and the supernatants were assayed for radioactivity.
23) are produced by polypeptide b. Also in agreement with the role of polypeptide b was the observation that, like in transformation (3, 6), acid-soluble oligonucleotides were produced from double-stranded DNA. Conceivably, the mononucleotides, nucleosides, and free bases which during transformation are released into the medium with the oligonucleotides result from secondary reactions by enzymes that are different from polypeptide b and are not necessarily essential for transformation.

Shortly after entry in *S. pneumoniae*, *S. sanguis*, and *B. subtilis*, the single-stranded donor DNA is associated with cellular proteins (ellipse complex; 11, 12, 16–18). It has been pointed out that the single-stranded binding proteins might fulfill a dual function (7, 12) (i) during DNA uptake, by facilitating binding and subsequent entry of the DNA, and (ii) after DNA entry, by protecting the single-stranded DNA against degradation by cellular nucleases. Evidence that polypeptide a plays a role in binding of donor DNA in *B. subtilis* has been discussed above. The results obtained so far did not give conclusive answers to the question whether polypeptide a might protect entered single-stranded DNA against degradation. Although the native 75,000-dalton protein complex bound strongly to single-stranded DNA, no binding activity of the separated polypeptide a to single-stranded DNA could be detected.

Another possible candidate for the *B. subtilis* eclipse protein is the competence-specific single-stranded DNA-binding protein described by Eisenstadt et al. (4). In contrast to polypeptide a, this protein is specific for single-stranded DNA and protects DNA in vitro against several nucleases, including DNase I.

The observed effects on polypeptide b, DNase I, and micrococal nuclease indicated that polypeptide a affected interactions between DNA and nucleases. Although the mechanism remains to be elucidated, it is conceivable that polypeptide a plays a role in DNA entry. One possibility to explain the effect on polypeptide b is that polypeptide a affects conformational changes of the DNA, such as unwinding. Since polypeptide b is specifically directed toward double-stranded DNA, DNA unwinding might result in a reduction of the nucleolytic activity of polypeptide b.

The results and considerations presented in this and previous papers can be summarized in a tentative model for the processes of DNA binding and entry during transformation in *B. subtilis*. In competent cells the tetrameric 75,000-dalton protein complex is located in the cellular membrane (21). The presence of divalent cations results in nicking of one strand, presumably at or close to the sites of binding. Since polypeptide a did not show any nuclease activity, it is assumed that polypeptide b induces the initial nick. Evidence for this activity of polypeptide b was shown previously (21). The initial single-stranded break will be followed by a second break. Since binding fixes the donor DNA relative to the nuclease subunit b, the second break can only be introduced opposite, or nearly opposite, the first break. The action of polypeptide b on one of the strands produces oligonucleotides. At the same time, the complementary strand enters the cell.

The model shows many similarities with the model proposed for DNA binding and entry in *S. pneumoniae* (7). One difference is that in *S. pneumoniae* the initial single- and double-stranded breaks are supposed to be introduced by different enzymes. Another difference concerns the specificity of the entry nucleases. The entry nucleases of *B. subtilis* is specific for double-stranded DNA, whereas the *S. pneumoniae* entry nuclease is also active on single-stranded DNA (7). Therefore, once a segment of single-stranded DNA has been formed in *B. subtilis*, it will be resistant to polypeptide b. In *S. pneumoniae* the entering single-stranded DNA may require protection against the entry nuclease, for example, by the single-stranded binding protein observed in this organism (11, 12).

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