Interactions of *Bacillus subtilis* RNA Polymerase with Subunits Determining the Specificity of Initiation

SIGMA AND DELTA PEPTIDES CAN BIND SIMULTANEOUSLY TO CORE*

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The *Bacillus subtilis* RNA polymerase sigma subunit and the phage SP82 encoded 28-kDa peptide are responsible for the binding of RNA polymerase to early and middle SP82 promoters, respectively. The delta peptide enhances the specificity of the interaction of *B. subtilis* RNA polymerase with these promoters. We have used sedimentation experiments to determine the effect of each of the three specificity factors, delta, sigma, and the 28-kDa peptide, on the binding of the other two factors to RNA polymerase core and the effect of NaCl on these binding equilibria. We show that sigma and the 28-kDa peptide can each bind to RNA polymerase core at the same time as delta. Sigma and the 28-kDa peptide have similar affinities to core at 0.1 M NaCl, but the 28-kDa peptide binds to core-delta more strongly than sigma. The implications of these findings with respect to the replacement of sigma by the 28-kDa peptide and the mechanism of promoter search by *B. subtilis* RNA polymerase are discussed.

The RNA polymerases of most prokaryotes consist of a catalytic core (containing α, β, and β' subunits) and a sigma subunit. Sigma subunits specify the promoter sequence recognized and hence the initiation sites. In *Bacillus subtilis*, different sigma subunits are used during different stages of growth, sigma (henceforth referred to as sigma) being predominant during vegetative growth (reviewed in Ref. 1). Following infection with phage SP82 or the related phage SP01, new sigma subunits are synthesized: a 28-kDa peptide which recognizes genes activated in the middle of infection and two other peptides which are required for transcription of late genes (reviewed in Ref. 2). The host sigma factor continues to be present throughout phage infection, and the mechanism of its replacement by the phage peptides is unknown. *B. subtilis* RNA polymerase also contains the delta peptide which increases the specificity of initiation by the host RNA polymerase and the two phage-modified polymerases (3–5).

Sedimentation studies showed that addition of delta to RNA polymerase core containing sigma leads to the release of sigma from the core (6) but only core-sigma is bound to DNA (7, 8). Williamson and Doi (6, 8) therefore suggested that delta and sigma bind competitively to core and act sequentially during initiation. However, Achberger and Whiteley (3) showed that both delta and a sigma factor are required for specific initiation at a promoter and proposed that sigma and delta act simultaneously. We have re-examined the binding of delta and sigma to RNA polymerase core at different concentrations of NaCl and found that this binding is not competitive but negatively cooperative at high concentrations of NaCl. Thus, the initiating polymerase may contain both delta and sigma subunits. Studies with the 28-kDa peptide and sigma showed that these subunits bind with similar affinities to RNA polymerase core. However, the 28-kDa peptide binds more tightly than sigma to core-delta. The latter tighter binding may be one factor in the mechanism of the replacement of sigma by the 28-kDa peptide.

**MATERIALS AND METHODS**

Purification of RNA Polymerase—RNA polymerase was purified from *B. subtilis* 168 as described (4) with a minor modification in the DNA cellulose column procedure. In this step, concentrated fractions from a Bio-Gel column were diluted into buffer C containing 10 mM Tris-HCl, pH 7.9, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.2 mM dithiothreitol, and 18% glycerol to give a conductivity equal to buffer C + 0.15 M NaCl and applied to the column. The column was washed, and RNA polymerase was eluted stepwise with buffer C + 0.3 M NaCl to give core-delta, followed by buffer C + 0.6 M NaCl to elute core-sigma. The core-delta and core-sigma fractions were concentrated separately and applied to 22–34% glycerol gradients containing buffer C + 0.1 M NaCl for sedimentation at 37,000 rpm for 40 h in a SW 283 rotor of the B-60 International Centrifuge; this step separated free core from core-sigma or core-delta. The percentage saturation of sigma or delta in the fractions selected for the binding studies was determined by densitometry of Coomassie Blue-stained SDS-polyacrylamide gels comparing the intensities of these peptides to that of the α subunit of the core. The core-delta preparations were 90% saturated with delta while the core-sigma preparations were 100% saturated with sigma. Core-sigma had a specific activity of 2.1 pmol of UMP incorporated/h/mg when assayed at 37 °C in the presence of delta using 60 µg/ml RNA polymerase and 20 µg/ml SP82 DNA in 40 mM Tris-HCl, pH 7.9, 10 mM MgCl2, 50 mM NaCl, 1 mM EDTA, 1 mM [γ-32P]UTP, 10 mM CTP, 10 mM GTP, 10 mM ATP. The activity of core-delta fractions varied due to traces of sigma but could be simulated between 6- and 10-fold on addition of excess sigma to a specific activity similar to that of the core-sigma fractions.

RNA polymerase containing the 28-kDa peptide (middle polymerase) was purified from log phase cultures of *B. subtilis* 168 5 h after infection with SP82 using a purification procedure similar to that used for polymerase from uninfected cells. The DNA cellulose column was eluted with buffer C + 0.3 M NaCl to obtain core containing delta.
and the 28-kDa peptide and subsequently with buffer C + 0.45 M NaCl to obtain core with the 28-kDa peptide and an additional peptide of 18 kDa.

Sigma was purified from core-sigma by phosphocellulose chromatography at low salt (6). Delta and subunits were purified from core-sigma by phosphocellulose chromatography, and only relative quantities of the core subunits were used for comparing the binding of the three subunits. These relative quantities were converted to molar equivalents from the known amount of total subunit added using the following values: core polymerase, M, 380,000; \( \alpha \) M, 45,000; delta, M, 21,000; and sigma, M, 43,000 (15).

**RESULTS**

The binding of polymerase subunits to core was investigated using sedimentation experiments. Core-sigma or core-delta was preincubated with other subunits for 1 h at 0 °C to achieve equilibrium (10) in the association of subunits with the core, and the samples were centrifuged on glycerol gradients. The gradients were fractionated and analyzed by dot immunobLOTS to determine which subunits were associated with the core.

**Binding of Delta to Core-Sigma at 0.1 M NaCl**—Fig. 1 shows the effect of delta on the sedimentation of core-sigma under conditions similar to those used by Williamson and Doi (6). Gradient 1 is a control which contained core-delta while gradients 2–4 contained core-sigma with increasing amounts of delta. Panel A, probed with serum against the \( \beta \) subunit, shows that RNA polymerase core was found mainly in fractions 10–16 for both core-sigma and core-delta. As seen from panel B, probed with serum against delta, delta sedimented with the core both in the core-delta sample and on addition of delta to core-sigma. Free delta, detected in fractions 1–4, was only observed when more than 1 eq of delta was added (gradient 4). Panel C, probed with serum against sigma, shows that sigma dissociated from the polymerase core even in the absence of delta (gradient 2). As observed by Williamson and Doi (6), in the presence of delta, most of the sigma was released from core (fractions 3–6); however, traces of sigma were still found in fractions 12–24. The addition of sigma had little effect on the sedimentation of core-delta at 0.1 M NaCl; the added sigma remained at the top of the gradient (data not shown).

These experiments show that delta binds more tightly to core than does sigma under these conditions. However, the effect of delta on the binding of sigma to core could be explained by either direct competition or negative cooperativity between the subunits. In simple competition, the subunits bind to the same site on RNA polymerase so that as one subunit is added the other is displaced. In negative cooperativity, on the other hand, the subunits bind at different sites so that both can bind simultaneously, but one weakens the binding of the other. In this case, as one subunit is added, the apparent binding constant of the other decreases to a plateau value, characteristic of the binding constant for the formation of the ternary complex.

**Effect of Solution Conditions on the Association of Sigma and Delta with Core**—A number of different solution conditions were tested to determine their effect on the sedimentation of core-sigma or core-sigma with added delta. Both sigma and delta bound more tightly to core at pH 6.6 than at pH 7.6 or 8.6 (data not shown). The presence or absence of 10 mM MgCl\(_2\) had no detectable effect on the binding of either subunit to core (data not shown); however, the concentration of NaCl had a differential effect on the binding of the subunits to core. Fig. 2 shows the amount of sigma or delta expressed as percent of the total subunit in each fraction of glycerol gradients of core-sigma (panel A) and core-delta (panel B) sedimented at three concentrations of NaCl. The standard curves used for the determination of the concentrations of sigma and delta in each fraction are shown as insets in the
FIG. 1. Sedimentation of core-delta and core-sigma supplemented with increasing quantities of delta at 0.1 M NaCl. Core-delta or core-sigma supplemented with delta were sedimented through glycerol gradients and fractionated into 0.5-ml fractions. Samples from each fraction of the gradient were spotted from left (top) to right (bottom) and analyzed using antibodies as described under "Materials and Methods." Gradient 1, 70 μg of core-delta; gradient 2, 70 μg of core-sigma; gradient 3, 70 μg of core-sigma plus 0.75 eq of delta; gradient 4, 70 μg of core-sigma plus 1.5 eq of delta. Panel A, 15-μl samples probed with antibodies to the β subunit; Panel B, 60-μl samples probed with antibodies to delta; Panel C, 20-μl samples probed with antibodies to sigma.

FIG. 2. Sedimentation of core-sigma and core-delta at different salt concentrations. Core-sigma (Panel A) and core-delta (Panel B) were sedimented through glycerol gradients at 0.1 (solid lines), 0.2 (dashed lines), and 0.3 (dotted lines) M NaCl. 0.5-ml fractions were taken from each, and samples from each fraction were spotted. The amount of subunit in each fraction was determined by densitometry of immunodots as described under "Materials and Methods" and expressed as a percentage of the total. Core subunits were detected in fractions 9–13. The inset in each figure shows the curve obtained on densitometry of duplicate standard samples of both free and bound subunits. The dotted boxes show the regions of the curves used in quantitation of the experimental samples.

The polymerase core sedimented slightly more slowly at 0.3 M NaCl than at 0.1 M NaCl in both samples. This may be due either to a change in conformation of the core or to aggregation at the lower NaCl concentration as found for Escherichia coli RNA polymerase (16). Panel A shows that there was release of sigma from core at 0.1 M NaCl (fractions 2–8) as seen in the previous figure. The amount remaining with the core (fractions 9–13) differed somewhat in different experiments ranging from 65 to 80% of the total. As the concentration of NaCl was increased, there was a slight increase in the amount of sigma released from core. At 0.3 M NaCl, approximately 55% remained bound. Panel B shows that little delta dissociated from the core at 0.1 M NaCl (fractions 1–8), in agreement with the results in Fig. 1 in which it was shown that delta binds more tightly to core than sigma. At higher concentrations of NaCl there was a sharp decrease in the amount of delta bound to core, so that at 0.3 M NaCl approximately 55% remained bound (fractions 9–14). Thus, at 0.3 M NaCl, delta and sigma bind to core with similar affinity.

Association of Sigma and Delta with Core at 0.3 M NaCl—Table I shows the effect of the addition of delta to core-sigma at 0.3 M NaCl. In this table, the amounts of bound and free subunits were calculated in terms of molar equivalents, based on the percentage of each and the total amount of subunit placed on the gradient. The core-sigma samples were initially saturated with sigma while the core-delta samples contained 0.9 eq of delta. For core-sigma, in the absence of delta, 0.69 eq of the sigma was bound to core. In the presence of 1 eq of delta, slightly more sigma was released and 0.45 eq of delta was bound to the core. Addition of increasing amounts of delta (up to 10 eq) led to increased binding of delta but to little further release of sigma. Conversely, when sigma was added to core-delta (gradient 2), there was little release of delta although the sigma bound to the core. These data show that sigma and delta do not bind competitively to core at 0.3 M NaCl but either independently or with a slight negative cooperativity. The two peptides are thus able to bind simultaneously.

The Binding of the 28-kDa Peptide to Core-Sigma and Core-Delta—We next determined the effect of the 28-kDa peptide on the association of sigma and delta with core. As shown in Fig. 3A, as increasing amounts of 28-kDa peptide were added to core-sigma, more 28-kDa peptide was bound (solid circles) with the simultaneous release of sigma from core (solid

| TABLE I |
| Sedimentation of core + sigma + delta at 0.3 M NaCl |
| Sigma | Delta |
|---|---|
| Bound | Released | Bound | Released |
| Gradient 1 | | | |
| Core-sigma | 0.69 | 0.31 |  |  
| + delta, 1 eq | 0.56 | 0.54 | 0.45 | 0.55 |
| + delta, 3 eq | 0.55 | 0.45 | 0.64 | 2.36 |
| + delta, 10 eq | 0.53 | 0.47 | 0.74 | 9.26 |
| Core-delta | 0.62 | 0.38 |  |  
| Gradient 2 | | | |
| Core-delta | 0.53 | 0.47 |  |  
| + sigma, 1.5 eq | 0.50 | 1.0 | 0.54 | 0.47 |
| + sigma, 4.0 eq | 0.80 | 3.2 | 0.63 | 0.37 |

*Expressed as equivalents.
The amount of the 28-kDa peptide (circles) and sigma or delta (squares).

In Panel A, the dotted line shows the theoretical curve for two peptides of similar affinity binding competitively. In Panel B, the dotted line shows the titration curve for a $K_d$ of $1.7 \times 10^5$ M$^{-1}$.

Again, the binding of sigma and the 28-kDa peptide could be either competitive or negatively cooperative; however, since sigma and the 28-kDa peptide have analogous and mutually exclusive functions, it seems reasonable to assume that they bind competitively. The dotted lines show the theoretical curves for the competitive binding of sigma and the 28-kDa peptide to core, assuming that they have similar affinities to core and that the core is initially saturated. In this simple case, $\sigma_b = \sigma_{total}/(\sigma_{total} + 28$ kDa$_{total}$) and similarly for the 28-kDa peptide bound. The experimental points fall close to these lines.

Fig. 3B shows the effect of the 28-kDa peptide on the association of delta with core. As more 28-kDa peptide was added, increasing amounts bound to the core (solid circles); however, the amount of delta bound to the core (solid diamonds) remained constant. Calculations using the observed amounts of bound and released 28-kDa peptide and known initial concentration of core-delta in two different sets of gradients gave apparent association constants of $1.1-2.6 \times 10^5$ M$^{-1}$. The dotted line in the figure shows the theoretical binding curve for an association constant of $1.7 \times 10^5$ M$^{-1}$.

The initial points for the 28-kDa peptide binding follow this curve. This association constant is probably lower than the true one due to release of some of the initially bound 28-kDa peptide during sedimentation (17).

Effect of NaCl on the Binding of the 28-kDa Peptide to Core-Sigma and Core-Delta—On sedimentation of core-sigma + 0.8 eq of the 28-kDa peptide at increasing concentrations of NaCl, the amount of sigma bound to core decreased while the amount of the 28-kDa peptide bound to core increased slightly (Table II). Thus, at higher concentrations of NaCl, the 28-kDa peptide binds more tightly to core than does sigma. However, when the experiment was performed with core-delta plus the 28-kDa peptide, increasing amounts of delta dissociated from the core, but the amount of the 28-kDa peptide bound remained unchanged (data not shown).

Effect of Delta on the Equilibrium between Core-Sigma and the 28-kDa Peptide—As shown in Fig. 1, at 0.1 M NaCl, sigma did not bind appreciatively to core-delta whereas Fig. 3 shows that the 28-kDa peptide was bound. Therefore, in a mixture of core, sigma, and the 28-kDa peptide, the addition of delta should cause the preferential release of bound sigma. Fig. 4

![Figure 3](image1.png)

**Fig. 3.** Sedimentation of core-sigma and core-delta with increasing amounts of the 28-kDa peptide. Increasing amounts of the 28-kDa peptide were added to either core-sigma (Panel A) or core-delta (Panel B) and sedimented through glycerol gradients. The amount of each subunit in each fraction was determined by densitometry of immunodots as described under "Materials and Methods." The amount of the 28-kDa peptide (circles) and sigma or delta (squares or diamonds) bound associated with the core is plotted against the amount of the 28-kDa peptide added. In Panel A, the dotted line shows the theoretical curve for two peptides of similar affinity binding competitively. In Panel B, the dotted line shows the titration curve for a $K_d$ of $1.7 \times 10^5$ M$^{-1}$.

![Table II](image2.png)

**Table II**

| NaCl (M) | Sigma Bound (eq) | Sigma Released (eq) | b/P | 28-kDa peptide (eq) | 28-kDa Released (eq) | b/P |
|---------|------------------|---------------------|-----|---------------------|---------------------|-----|
| 0.1     | 0.62             | 0.38                | 1.5 | 0.38                | 0.42                | 1.0 |
| 0.2     | 0.28             | 0.62                | 0.6 | 0.43                | 0.57                | 1.2 |
| 0.3     | 0.22             | 0.78                | 0.3 | 0.47                | 0.33                | 1.4 |

* Ratio bound/released.
shows that when core-sigma plus 1 eq of the 28-kDa peptide (open circles) was sedimented in the absence of delta, about half of each subunit was bound. On addition of 2 eq of delta (solid circles), very little sigma remained bound (panel A), but the binding of the 28-kDa peptide (panel B) was unaffected. Similar results were observed at 0.2 and 0.3 M NaCl (data not shown).

Discussion

Sigma and Delta Bind Simultaneously to Core-Delta—The experiments in Table I show that sigma and delta do not bind competitively to B. subtilis RNA polymerase core at 0.3 M NaCl and, therefore, simultaneous binding is possible and, in fact, both subunits are bound. It therefore seems probable that they can also bind simultaneously at 0.1 M NaCl. The results obtained in Fig. 1 at 0.1 M NaCl show that the binding of delta to core decreases that of sigma, i.e. delta and sigma bind with strong negative cooperativity. The binding of sigma must, therefore, also decrease the binding of delta to core. However, little effect of sigma on core-delta was observed at 0.1 M NaCl primarily because the binding of sigma in the presence of delta is weak so that little sigma is bound unless an excess is added. Second, even though the binding constant of delta is decreased by the presence of sigma, it may still be sufficiently high for most of the delta to be bound at the concentrations of core and delta used in these experiments. As the concentration of NaCl increases, the binding of sigma and delta to core decreases and so does the negative cooperativity between them. Thus, at 0.3 M NaCl little effect of either subunit on the binding of the other is observed.

Effect of NaCl—The above effects of NaCl on the binding of sigma and delta to core could be due either to ionic interactions which are shielded at high concentrations of NaCl or to changes in the conformation of the RNA polymerase induced by NaCl. Such changes in the conformation of B. subtilis RNA polymerase with NaCl have been demonstrated using chymotrypsin digestion of core-sigma (18). The latter work showed that an increase in the NaCl concentration from 50 to 300 mM led to slower degradation of the beta and sigma subunits, without affecting the rate of degradation of a control protein, beta-galactosidase. Ions have also been reported to affect the conformation of E. coli RNA polymerase (16). Such changes in the conformation of RNA polymerase with NaCl concentration could weaken the binding of the delta and sigma subunits and lessen the negative cooperativity between them. The binding of subunits to core could also influence conformation, thereby affecting negative cooperativity. Evidence for such conformational changes has been obtained in experiments on the photochemical cross-linking of B. subtilis RNA polymerase to DNA (19) and in methylation protection experiments (20).

Binding of the 28-kDa Peptide—The results of Fig. 3A suggest that sigma and the 28-kDa peptide bind competitively to core, i.e. that they bind at the same binding site and with similar affinities at 0.1 M NaCl. At higher concentrations of NaCl, the 28-kDa peptide binds more tightly than sigma (Table II). In the presence of delta, the binding of the 28-kDa peptide to core appears to be insensitive to the concentration of NaCl; thus, the apparent increase in the binding of the 28-kDa peptide at high concentrations of NaCl in the presence of sigma is probably largely due to the weaker binding of sigma to core. The relative affinities of sigma and the 28-kDa peptide to core as determined in these experiments are in general agreement with experiments of Chelm et al. (10) in which transcription was used to measure the binding of sigma and gp28 (from phage SP01 which is closely related to phage SP82) to free core.

The present experiments also show that in the presence of delta, the 28-kDa peptide is bound to core in preference to sigma (Fig. 4). The addition of delta to core-sigma + 28-kDa peptide mixture does not lead to an increase in the amount of the 28-kDa peptide bound to the core although sigma and the 28-kDa peptide bind competitively to core and most of the sigma is displaced. This indicates that there is some negative cooperativity between the 28-kDa peptide and delta although the degree of this negative cooperativity is clearly less than that between sigma and delta. The difference in the behavior of sigma and the 28-kDa peptide in the presence of delta may be due in part to the smaller size of the 28-kDa peptide. If the 28-kDa peptide binds to only part of the sigma binding site, delta could affect another part of this site. A difference in the binding of sigma and the 28-kDa peptide to core-delta could lead to increased transcription of middle promoters relative to early promoters during phage development. However, our equilibrium results contrast with the kinetic experiments of Chelm et al. (10) on gp28. The latter investigators found that when sigma and gp28 were added to core for 2.5 min before initiation of transcription, the addition of delta shifted the ratio of transcripts directed by sigma or gp28 in favor of sigma, rather than gp28. This indicates that delta increased the rate of association of sigma to core relative to that of gp28. The kinetic and equilibrium results can be reconciled by postulating that delta increases the ratio of the dissociation rates of sigma and the 28-kDa peptide from core more than it increases the ratio of their association rates. Further experiments are required to determine whether it is the association rates or the association constants of the subunits to the core than affect transcription of different promoters.

The finding that delta and sigma peptides can bind simultaneously to RNA polymerase core resolves a problem in the mechanism of promoter search by B. subtilis. Based on the idea that the binding of delta and sigma to core are mutually exclusive, Williamson and Doi (6, 8) suggested that core-delta binds at promoters with the release of delta, followed by the binding of sigma, thus allowing initiation to occur. This model seems unlikely to be correct as core-delta does not form complexes that are stable to filter binding with SP82 DNA (3), and delta enhances the specificity of polymerase with different sigma factors, each recognizing a different promoter sequence (3). RNA polymerase lacking delta binds and initiates at both promoter and nonpromoter sites (3); thus, delta is necessary to discriminate against nonpromoter sites while sigma is necessary for binding to DNA. Spiegelman and Whiteley (7) proposed an alternative model, in which sigma and delta bind simultaneously to core, directing the promoter search. This study supports the latter model by showing that simultaneous binding of delta and sigma to core RNA polymerase can occur. The high concentrations of polymerase, delta and sigma in the cell should promote the simultaneous binding of the two subunits despite their negative cooperativity. The negative cooperativity may be of importance either in later stages of RNA synthesis when sigma and delta are released from core or in the replacement of one sigma subunit by another.

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