Kinetics of Cross-Linking of Peptidoglycan in *Bacillus megaterium*

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SUMMARY

The extent and rate of cross-linking of diaminopimelic acid in the peptidoglycan of *Bacillus megaterium* have been determined by a new procedure. The method is based on the reaction of nitrous acid with the unprotected amino groups of non-cross-linked diaminopimelic acid.

Pulse label experiments in a mutant where diaminopimelic acid is incorporated specifically into peptidoglycan demonstrated that the cross-linking reaction continued for many minutes after incorporation of precursor subunits into peptidoglycan. The method has been used to demonstrate the sensitivity of the cross-linking reaction to penicillin in vivo. Models for assembly of peptidoglycan are discussed.

At present, information on the extent of cross-linking is obtained by preparation of cell walls, treatment with 2,4-dinitrofluorobenzene and separation of derivatized and underivatized DAP$^+$ after hydrolysis (7). In view of the use of a biphasic system for the derivatization reaction, it is perhaps not surprising that variable values for the extent of cross-linking have been obtained even in a single species (8, 9). Moreover, no information exists on the rate of cross-linking.

Clearly, a detailed knowledge of the cross-linking reaction is a prerequisite to an understanding of how the bacterial cell wall is assembled. In order to provide such information we have introduced a new procedure for determining cross-linking. This procedure is based on the reaction of nitrous acid with the amino groups of DAP which are not protected by cross-linking. The application of this procedure is facilitated with the use of a double auxotroph of *Bacillus megaterium* which requires DAP and is unable to degrade DAP to lysine. In this mutant strain exogenous radioactive DAP exclusively labels the DAP in the cell wall.

When labeled cells are treated with nitrous acid, the DAP molecules with amino groups not protected by cross-linking are converted to hydroxyaminopimelic acid. After hydrolysis, the radioactive DAP which had been cross-linked and HAP formed from non-cross-linked DAP can be separated by paper chromatography or ion exchange to give a measurement of the extent of cross-linking.$^2$ By exposing the double auxotroph to pulses of radioactive DAP it has been possible to obtain information about the rate of the cross-linking reaction. This knowledge of the kinetics of assembly of peptidoglycan has provided insight into the mechanism of formation of cell walls during cellular growth.

A preliminary account of this work has appeared (10).

METHODS AND MATERIALS

Bacterial Strain and Growth Condition—All experiments were carried out with the mutant strain of *B. megaterium* M-46 (DAP, *Lys*). Conditions for the growth and properties of the mutant have been previously described (11, 12). A minimal medium supplemented with DAP, 5 $\mu$g per ml, and L-lysine-HCl, 10 $\mu$g per ml, was used for growth of the mutant strain.

Incorporation of [^14C]DAP into Peptidoglycan—For determination of the maximum extent of cross-linking in exponential phase and stationary phase cells, labeled DAP was present throughout the growth phase. For pulse experiments where the rate of cross-linking was determined, the radioactive label was added when the culture was in the exponential phase of growth. The pulse experi-

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1 The abbreviations used are: DAP, diaminopimelic acid; HAP, hydroxyaminopimelic acid.

2 Preliminary experiments demonstrating the feasibility of the procedure were carried out by Linda Nussbaum.
experiments were quenched by adding a hundred times excess of unlabeled DAP.

Cell growth was stopped by the addition of aliquots of the culture to equal volumes of 8% (w/v) sodium dodecyl sulfate. The labeled cells were harvested by centrifugation at room temperature for 15 min at 10,000 rpm. The cells were washed by suspension in water followed by centrifugation. The washing procedure was repeated (usually six times) until the supernatants were free of radioactivity.

In order to determine the extent of incorporation of radioactivity, cells were collected either by filtration of a 0.1-ml aliquot of the suspension of cells in 4% sodium dodecyl sulfate through a 0.45-μm pore size Millipore membrane filter followed with two 5-ml portions of water, or by washing the cells free of unincorporated DAP by centrifugation as described above, and sampling directly. The washed cells were resuspended in 3.0 ml of H2O. Radioactivity was measured in a Packard model 3003 Tri-Carb liquid scintillation spectrometer after dissolving the membrane filters or adding a 30-μl aliquot of the 3.0-ml suspension to 10 ml of the scintillation fluid described by Bray (13).

**Determination of Cross-Linking in Peptidoglycan**—The method for determination of cross-linking was based on the reaction of free amino groups with nitrous acid (14). An appropriate fraction of the sodium dodecyl sulfate-treated cells (1.5 to 2.0 mg dry weight for the experiments of Table I; 0.1 to 0.4 mg dry weight for the kinetic experiments) containing [14C]DAP was suspended in 3 ml of H2O and the tubes were placed in an ice bath. Sodium nitrite (0.1 g, final concentration =0.5 M) and glacial acetic acid (0.15 ml, final concentration =0.9 N) were added. The samples were kept at 0° for 11/2 to 2 hours. The cells were collected by centrifugation for 10 min at 10,000 rpm at 0°. The pellet was then washed by resuspension in 10 ml of H2O and centrifugation. This was repeated three times.

In order to facilitate complete hydrolysis, the cells that had been treated with nitrous acid were treated with lysozyme before hydrolysis in HCl. The pellets from the last washing were suspended in 0.5 ml of 0.001 M phosphate buffer, pH 6.3, containing lysozyme, 0.05 mg per ml, and placed in a 32° water bath for 2 hours.

The samples were then hydrolyzed in 1.0 to 2.0 ml of 6 N HCl in closed tubes for 4 hours in an autoclave at 125°. The hydrolyzed solutions were evaporated to dryness on a water bath to remove the HCl, then redissolved in 1.0 ml of H2O. This procedure was repeated three times.

The radioactive products obtained from the peptidoglycan of the cell wall by hydrolysis were DAP that had been cross-linked in the wall, and α-hydroxy-α-aminopimelamic acid formed by the nitrous acid reaction from non-cross-linked DAP. These products were separated by descending paper chromatography on Whatman No. 1 filter paper in methanol-water-pyridine (80:20:10), the solvent system of Ruhland et al. (15). The residue remaining after HCl had been removed from the sample by evaporation was dissolved in either 5 μl or 10 μl of H2O, and the entire sample was spotted on the paper. After developing and drying, the paper was cut into 0.5-cm sections and each piece was counted in 10 ml of Bray’s scintillation fluid. The per cent cross-linking was determined by dividing the total counts in the peak corresponding to DAP by the sum of the counts in the peaks corresponding to DAP and HAP. Radioactivity which remained after HCl had been removed from the sample by evaporation was determined by dividing the total counts in the peak corresponding to DAP by the sum of the counts in the peaks corresponding to DAP and HAP. Radioactivity which remained on the paper chromatograph (usually less than 4%) was ignored. An example of the distribution of radioactive products on the paper is shown in Fig. 1. In some experiments the radioactive products were separated by ion exchange on an anion exchange resin (Bio-Rad AG 1-X2). At neutral pH, α-hydroxy-α-aminopimelamic acid is retained on the column, while DAP is eluted by H2O. For the ion exchange separation, the samples after hydrolysis and evaporation were redissolved in 0.5 ml of H2O. The solution was adjusted to pH 7 with KHCO3, a 0.1-ml aliquot was taken to determine total counts, and a 0.2-ml aliquot was added to a column containing about 0.5 ml of the anion exchange resin. The column was washed with 1.3 ml of H2O. The entire column effluent was collected in a scintillation vial. In order to have comparable counting conditions, 1.4 ml of water were added to the 0.1-ml aliquot. Both samples were counted in 10 ml of Bray’s scintillation fluid. The per cent cross-linking was calculated by dividing one-half of the counts in the column effluent by the total counts in the 0.1-ml aliquot.

**RESULTS**

**Cross Linking in B. megaterium**—The first application of the nitrous acid procedure was to determine the extent of cross-linking in exponential phase and in stationary phase cells. As described under "Methods and Materials," [14C]DAP was added to the culture medium before inoculation to ensure uniform labeling. The growth of this culture was limited by the glucose concentration (0.1%). The amount of cross-linking determined by the nitrous acid procedure was found to be 50 and 56%, respectively, for exponential and stationary phase walls (Table I).

**Kinetics of Cross-Linking**—The increased degree of cross-linking obtained with stationary phase cell walls compared to exponential phase cell walls already suggested that the cross-linking reaction did not proceed instantaneously with the incorporation of the DAP into the peptidoglycan. In order to explore the kinetics of the cross-linking reaction more fully, experiments were set up in which pulses of isotopically labeled DAP were administered to cells.

**TABLE I**

**Cross-linking of DAP in B. megaterium M-46**

<table>
<thead>
<tr>
<th></th>
<th>DAP cross-linked</th>
</tr>
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<tr>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Exponential phase cells</td>
<td>60</td>
</tr>
<tr>
<td>Stationary phase cells</td>
<td>56</td>
</tr>
</tbody>
</table>

Bacteria were grown in 25 ml of minimal salts medium containing DAP, 5 μg per ml, lysine-HCl, 10 μg per ml, and 1.0 μCi of [14C]DAP in 300-ml Nephelo flasks at 32° with vigorous aeration. Exponential phase cells were obtained at a turbidity of 43 Klett units and stabilized by adding 15 ml of the culture to 15 ml of 8% sodium dodecyl sulfate; stationary phase cells were collected at a turbidity of 87 Klett units, 2½ hours after the end of exponential growth by diluting the remaining 10 ml with 10 ml of 8% sodium dodecyl sulfate. Cross-linking was determined using one-half of each sample (~1.5 to 2.0 mg dry weight) as described under "Methods and Materials." The radioactive products were separated by paper chromatography.
The experimental protocol for determining the rate of cross-linking is illustrated in Fig. 2. Exponentially growing cells were given a 5-min pulse of \textsuperscript{14}C]-DAP, followed by a 100-fold excess of unlabeled DAP to quench incorporation of the label, and samples were taken at 5-min intervals. As before, growth was stopped by suspending the cells in sodium dodecyl sulfate. Incorporation of \textsuperscript{14}C]-DAP into the cell was measured by Millipore filtration. Fig. 2 shows the effectiveness of the quenching. Most of the labeled DAP incorporation occurred during the period of the pulse. However, about 20\% additional incorporation took place in the 5 min after dilution with unlabeled DAP.

Measurements of the cross-linking are listed in Table II and show that although cross-linking was a fast reaction with a major portion being completed within the pulse period, cross-linking continued to increase 25 min after incorporation of DAP into the wall.

In order to examine the kinetics of cross-linking at early time periods more completely, isotope was administered in a 1-min pulse to exponential cells and samples were taken at 1-min intervals. As shown in Fig. 3, substantial incorporation of \textsuperscript{14}C]-DAP into the wall continued for about 3 min after quenching with a 100-fold excess of unlabeled DAP. This quenching stopped movement of \textsuperscript{14}C]-DAP into the metabolic pools, but several minutes were required to exhaust the label from the cytoplasmic pools of intermediates such as UDP-muramic acid-pentapeptide and the C\textsubscript{48} isoprenoid alcohol lipid carriers which are in the process of being incorporated into the peptidoglycan. As shown in Table III, cross-linking was most rapid in the first minute of the pulse and continued after incorporation of DAP into the cell wall. Despite the complication of continued incorporation, it is clear that not only can cross-linking occur subsequent to incorporation but that the bulk of the cross-linking takes place several minutes after the insertion of the subunit into the wall. This means that there is a maturing of the nascent cell wall which is related to this time-dependent formation of cross bridges.

**Penicillin Effects on Cross-Linking**—As a further illustration of the utility of this method, the rate of cross-linking was determined in the presence of penicillin. Penicillin is believed to affect growing cells through its ability to inhibit the transpeptidation reaction (5, 6). The effect of a low level (1 \(\mu\)g per ml) of penicillin on the growth of the \textit{B. megaterium} auxotroph is shown in Fig. 4. Cellular synthesis continued for a short time as indicated by an increase in turbidity, but at a greatly reduced rate, and then was completely inhibited. Incorporation of \textsuperscript{14}C]-DAP into the cell wall continued over this period. Fig. 4 also shows that the rate of incorporation of \textsuperscript{14}C]-DAP was linear for about 30 min and then gradually decreased. The extent of cross-linking in the presence of penicillin was determined by the nitrous acid

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**TABLE II**

**Cross-linking of DAP in \textit{B. megaterium} M-46, 5-min pulse**

<table>
<thead>
<tr>
<th>Sampling time (min)</th>
<th>DAP cross-linked (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>30</td>
</tr>
<tr>
<td>20</td>
<td>43</td>
</tr>
<tr>
<td>25</td>
<td>48</td>
</tr>
</tbody>
</table>

**TABLE III**

**Cross-linking of DAP in \textit{B. megaterium} M-46, 1-min pulse**

<table>
<thead>
<tr>
<th>Sampling time</th>
<th>DAP cross-linked (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
</tr>
<tr>
<td>4</td>
<td>29</td>
</tr>
<tr>
<td>5</td>
<td>33</td>
</tr>
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</table>
reaction. As shown in Table IV, penicillin almost completely inhibited the transpeptidase reaction.

This inhibition by penicillin provided an opportunity to test the independence of the insertion reaction and cross-linking. Exponential phase cells were pulse-labeled with [14C]DAP in the presence of penicillin. After 10 min, the penicillin was inactivated with penicillinase, and incorporation of [14C]DAP was quenched with excess unlabeled DAP. Although the cells did not recover from exposure to penicillin, i.e. growth did not resume, cross-linking of the labeled DAP which had been incorporated during the pulse increased 2-fold within 20 min.

**DISCUSSION**

The introduction of a new procedure for distinguishing between cross-linked and underivatized DAP coupled with the use of a DAP, lysine auxotroph has made possible for the first time an investigation of the kinetics of transpeptidation. It has been observed that the DAP moieties of entering disaccharide pentapeptide subunits participate in cross-linking in a reaction that reaches completion many minutes after the initial attachment to the cell wall. This finding provides a constraint in the light of which models for peptidoglycan assembly may be examined. In considering such models one must take into account the nature of the initial insertion reaction, the sequence of reactions that complete the polymerization and the mode of assembly of the subunits.

Each disaccharide pentapeptide presursor subunit has four possible attachment sites to the pre-existing peptidoglycan. These are the 1 carbon of the N-acetylmuramic acid, the 4-hydroxyl of the N-acetylglicosamine, the COOH end of the penultimate d-alanine (acting as donor in transpeptidation), and the amino group of the N half of the meso-DAP (acting as acceptor in transpeptidation).

In the simplest model of peptidoglycan synthesis, each subunit would always be attached to a growing point of peptidoglycan by the same one of the four possible bonds, i.e. the initial bond is exclusively of one type. The required insertion of every subunit via either N-alanine or DAP would produce a peptidoglycan with 100% cross-linking. This is not observed. It might be argued that 100% cross-linking is obtained initially and is reduced to the observed values by peptidase activity but such enzymes have not been reported to be present in the cell envelope. Moreover, the pulse experiments show that the degree of cross-linking increases with time rather than decreases. Therefore, this simplest model is consistent only with initial incorporation by addition to the glycan chain.

In the situation where the subunit enters the wall by forming a glycan bond, there remain two distinguishable sequences for the subunit to become involved in a cross-link. The first route obligatorily uses the DAP to form a cross-link with a subunit already in the wall. The second method uses the N-alanine in the same way. It may be that no cross-linking reaction can occur until some other change has taken place in the subunit such as formation of the second glycan bond or loss of the terminal N-alanine via the carboxypeptidase (16), but this aspect of the complete reaction sequence is not discerned by the nitrous acid method.

If the wall is assembled by random intercalation, the cross-linking cannot be achieved exclusively through use of the N-alanine of the new subunit since none of this type of cross-link would be scored by the nitrous acid method until the label had been thoroughly distributed throughout the wall. If this mechanism of wall assembly occurred, the kinetic experiments would exhibit a long lag before cross-linking is detected; this type of lag is not observed. Alternatively, for the case of random intercalation, if the cross-links were formed using only the DAP of the entering subunits, all cross-links involving the entering labeled subunit would be detected immediately by the nitrous acid method.

If the cross-links were formed using only the DAP of the entering subunits, all cross-links involving the entering labeled subunit would be detected immediately by the nitrous acid method. This mechanism for cross-linking exclusively via DAP of the entering subunit might fit the data but only if insertion and cross-linking are independent. If the reactions occurred simultaneously, the value of the per cent cross-linked DAP would be fixed at the maximum as soon as the subunits entered the peptidoglycan, and no buildup of cross-linking could occur. In fact, an increase in per cent DAP cross-linked as a function of time is observed.

If the peptidoglycan is assembled by incorporation of subunits into a growth zone, the observations can be explained by models in which the subunit enters by forming a glycan bond and then forms a cross-link. Either possibility for cross-link formation, i.e. exclusively via N-alanine, or exclusively via DAP, can fit the observed kinetics for established zones. Since each cross-link in an established growth zone involves two subunits, each with [14C]DAP of the same specific activity, the cross-linking is scored regardless of whether the entering subunit acts through either the DAP or N-alanine. Therefore, after labeling of the growth zone is established during a pulse, the method will not distin-
guish between cross-linking via DAP or d-alanine, but here, too, glycine chain formation and transpeptidation must be independent to be in agreement with the experimental observations.

It might be suggested that the delay in completion of cross-linking after incorporation of subunits is only an apparent delay, due to the time required to build up the labeled growth zone. Clearly this cannot be the case if cross-linking is exclusively through DAP acting as an acceptor since these cross-links score independent of their neighbors. Even the alternative case may be eliminated. Were d-alanine cross-linking and glycine insertion to occur simultaneously, then the DAP of the units initially added to the wall would register as incorporated, but the unscarable cross-links between the n-alanine and the unlabeled DAP of the peptidoglycan would create a deficit such that cross-linking in pulse-labeling experiments would never attain the same value as is measured in uniformly labeled cells. In point of fact, after 5 min the per cent cross-linking in a 1-min pulse is not very different from the value determined immediately after a 5-min pulse, and 20 min after a 5-min pulse the value is indistinguishable from results obtained in uniform labeling experiments.

It must be emphasized that most of the possibilities discussed above are valid only for the limiting cases where one specific bond is always the initial point of attachment of a subunit.

In a more complicated model of the reaction sequence, some of the subunits could enter by glycine bond formation, while the rest enter by forming a peptide cross-link. The latter would be a good method for originating new glycine chains, and the in vitro studies of Mirelman et al. (17) with Micrococcus lysodeikticus have provided evidence for such a reaction. It is clear from our results, however, that in B. megaterium no major fraction of the subunits is incorporated via the acceptor DAP. By extrapolating back to the initial period of a pulse (Table III), it can be seen that less than 10% of the incorporated DAPs are involved immediately in a cross-link. It should be noted again that incorporation via DAP is undetectable.

The pathway which best fits the experimental results on the extent and rate of cross-linking is initial incorporation of subunits by the formation of a glycine bond, followed at a later time by a transpeptidation involving either DAP or DAP of the entering subunit. Additional information will be required to determine if subunits enter in a growth zone or by random intercalation. Several of the possible reaction sequences can be eliminated if incorporation does occur by random intercalation. The kinetic method is less restrictive for assembly in growth zones. In either random or zonal assembly, however, transpeptidation and transglycosylation must be separate and independent reactions.

These results suggest a model for the action of the transpeptidase. This enzyme and the transglycosylase act independently. The transpeptidase can find and move to its substrates after incorporation of the subunits. It can catalyze the formation of cross-links at any time and at any location where the reactive groups (DAP and d-Ala-d-Ala) are in the required stereochemical orientation.

Additional evidence for this hypothesis is provided by the

Fig. 5. Cross-linking of DAP incorporated in the presence of penicillin. Bacteria were grown in 10 ml of minimal medium supplemented with DAP and lysine. At Klett 55, 30 μg of penicillin were added, followed after 1 min by 10 μCi of [14C]DAP. After 10 min (0 min on figure), 600 units of penicillinase and 5 mg of DAP were added. Immediately thereafter a 2.0-ml sample was taken. Similar samples were taken at 5-min intervals. O, incorporation of DAP determined by counting aliquots of washed samples; •, cross-linking determined as described under "Methods and Materials." DAP and HAP were separated by anion exchange chromatography.

REFERENCES
Kinetics of Cross-Linking of Peptidoglycan in *Bacillus megaterium*
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