Biosynthesis of Uridine Diphosphate N-Acetylmannosaminuronic Acid in rff Mutants of Salmonella typhimurium

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In Salmonella typhimurium, three groups of genes located in rfb, rfe, and rff clusters are known to be involved in the biosynthesis of the enterobacterial common antigen. We found that the enzymatic synthesis of uridine diphosphate N-acetylmannosaminuronic acid, the activated form of a constituent sugar of the common antigen, followed the pathway previously described in Escherichia coli (N. Ichihara, N. Ishimoto, and E. Ito, FEBS Lett. 39:46–48, 1974). All of the six rff mutants tested, which fail to synthesize the common antigen, were deficient in one or both of the two enzymes needed for the synthesis of this sugar nucleotide from uridine diphosphate N-acetylglicosamine; these results establish the physiological role of the pathway studied for the biosynthesis of N-acetylmannosaminuronic acid residues. The levels of these enzymes were not reduced in rfe mutants or rfb deletion mutants, although they produced no or only traces of the common antigen.

The enterobacterial common antigen (ECA) was discovered in Escherichia coli by Kunin in 1962 (5). It is found in many species of bacteria belonging to the family Enterobacteriaceae (8). It has been found to exist in two forms, an immunogenic form and, more commonly, a haptenic non-immunogenic form (8). Mayer has found that the latter form was a glycolipid. Two sugars, mannosaminuronic acid and glucosamine, each constitute about 25% of the weight (8). Palmitic acid (C16:0) was also present.

It has been recently shown that two gene clusters, rfe and rff, are involved in the biosynthesis of ECA (10). Both rfe and rff are linked to iut at min 122 on the Salmonella chromosome map. In group B Salmonella such as Salmonella typhimurium, a gene(s) in the rfb region (located at min 65) is also required (Fig. 1). Thus extended deletions in the rfb gene cluster of S. typhimurium LT2 produce strains with trace amounts of ECA (10). In addition, these strains were unstable, and during storage secondary mutations at the rff locus accumulated, completely abolishing the synthesis of ECA. Although rff mutations map very close to the rfe locus, they are different in the sense that the former affects only ECA biosynthesis whereas the latter is also necessary for the biosynthesis of various other polysaccharides such as O-side chains in group L and group C, Salmonella, and T, side chains (8, 9). A prime candidate for the function of rff is the synthesis of mannosaminuronic acid, a unique component of ECA. Mannosaminuronic acid has been found in the cell wall of Micrococcus lysodeikticus (14), in the capsular material of some E. coli strains, and in the cell wall of Staphylococcus aureus (17). Ichihara et al. have shown that the nucleotide derivative of mannosaminuronic acid, uridine diphosphate N-acetylmannosaminuronic acid (UDP-ManNacUA), is synthesized from uridine diphosphate N-acetylglicosamine (UDP-GlcNAc) in E. coli O14 (3). The pathway involves two enzymes, an epimerase which converts UDP-GlcNAc to uridine diphosphate N-acetylmannosaminuronic acid (UDP-ManNac) and a dehydrogenase which oxidizes the UDP-ManNac to UDP-ManNacUA using oxidized nicotinamide adenine dinucleotide (NAD\(^+\)) as a cofactor. In this study we measured the activity of these two enzymes in various mutants and discovered that the rff gene cluster is indeed necessary for these activities.

MATERIALS AND METHODS

Bacteria. All strains were derived from S. typhimurium LT2.

Preparation of soluble enzyme fraction. Bacteria were grown in L-broth (1) at 37°C with aeration by shaking, harvested by centrifugation at late exponential phase of growth, washed once with ice-cold water, and resuspended in 1/100 original volume of...
High-voltage paper electrophoresis. Sheets of Whatman 3MM paper were washed with water and dried at room temperature before use. A flat-bed electrophoresis apparatus (Savant) was used. All operations were done at 15°C. The systems used were: solvent E, pyridine-acetic acid-water (10:4:86, vol/vol/vol) (pH 5.4); solvent F, 1.0 M sodium molybdate (adjusted to pH 5.0 with H₂SO₄); solvent G, 0.05 M sodium tetraborate (pH 10.0).

Chemicals. UDP-[U-¹⁴C]GlcNAc was obtained from New England Nuclear. UDP-GlcNAc, NAD⁺, d-glucosamine, N-acetyl-d-mannosamine (ManNAc), and N-acetyl-d-glucosamine (GlcNAc) were all obtained from Sigma.

Purified ECA (8) was a gift of H. Mayer. ECA was hydrolyzed in 4 N HCl for 1 h at 100°C. The hydrolysate was evaporated over KOH pellets under vacuum, dissolved in water, and used as a standard in paper electrophoresis.

Enzyme assay methods. (i) Synthesis of UDP-ManNAcUA from UDP-GlcNAc. The procedure is essentially that used by Ichihara et al. (3), except for two modifications: the addition of MgCl₂ (2 mM) and carrier UDP-GlcNAc (0.5 mM). The reaction mixture, containing 450 µg of protein, was incubated at 37°C for 30 min.

(ii) Synthesis of UDP-ManNAc from UDP-GlcNAc. The reaction mixture contained, in a total volume of 0.1 ml: Tris-hydrochloride buffer (pH 7.9), 9 µmol; MgCl₂, 200 nmol; UDP-GlcNAc, 80 nmol; UDP-[U-¹⁴C]GlcNAc (160,000 cpm), 3 nmol; and soluble enzyme (180 µg of protein). After incubation at 37°C for 30 min, the reaction was stopped by boiling for 1 min. The protein precipitate was removed by centrifugation, and the supernatant was chromatographed in solvent A for 24 h at 21°C. The UV-absorbing region corresponding to UDP-ManNAc (R<sub>UDP-glcNAc</sub> = 1.00) was eluted with water and lyophilized. The material was hydrolyzed in 0.01 N HCl for 15 min at 100°C and subsequently evaporated to dryness under vacuum over KOH. ManNAc and GlcNAc were added at 100 nmol each as carriers. The mixture was subjected to paper electrophoresis in solvent G for 2 h at 32 V/cm. Bands corresponding to ManNAc and GlcNAc were detected by spraying the paper with the reagent of Morgan and Elson as described by Neuberger and Marshall (12). Under the conditions described, ManNAc and GlcNAc moved 10 and 4.5 cm, respectively, towards the anode. The ManNAc spot was cut, and the radioactivity in it was quantitated by liquid scintillation counting.

(iii) Synthesis of UDP-ManNAcUA from UDP-ManNAc. The oxidative step was measured by using, as substrate, a boiled reaction mixture containing a mixture of UDP-[U-¹⁴C]ManNAc and UDP-[U-¹⁴C]-GlcNAc.

Step I. The reaction mixture contained, in a final volume of 72 µl: Tris-hydrochloride buffer (pH 7.9), 9 µmol; MgCl₂, 200 nmol; UDP-[U-¹⁴C]GlcNAc (187,500 cpm), 0.6 nmol; UDP-GlcNAc, 50 nmol; and [his-⁵₁⁹]sulfolactate (450 µg of protein). After incubation at 37°C for 30 min, the reaction was terminated by boiling for 1 min. The protein precipitate was removed by centrifugation, and the supernatant was used in step II.
Step II. The reaction mixture contained, in a final volume of 120 μl: 65 μl of step I supernatant mixture (153,000 cpm); NAD⁺, 50 nmol; and soluble enzyme extract (450 μg of protein). After incubation at 37°C for 30 min, the reaction was terminated by boiling for 1 min. The protein precipitate was removed by centrifugation, and the supernatant was chromatographed for 24 h in solvent A. The region (R_{UDP-GlcNAc} = 0.55) corresponding to UDP-ManNAcUA was cut, and the radioactivity was determined by liquid scintillation counting.

RESULTS

Synthesis of UDP-ManNAcUA from UDP-GlcNAc. In preliminary experiments, we found that UDP-GlcNAc was converted into a UV-absorbing compound, nucleotide-X, when incubated with soluble enzyme fractions of ECA⁺ S. typhimurium. Nucleotide-X was made on a preparative scale. The reaction mixture contained, in a final volume of 0.2 ml: Tris-hydrochloride (pH 8.6), 10 μmol; UDP-[U-14C]-GlcNAc (120,000 cpm), 2.2 nmol; UDP-GlcNAc, 400 nmol; MgCl₂, 0.8 μmol; NAD⁺, 160 nmol; and S. typhimurium LT2 enzyme, 2.6 mg of protein. Four tubes, each containing the above mixture, were incubated at 37°C for 30 min. After the reaction was terminated by boiling for 1 min, the protein precipitate was removed by centrifugation, and the supernatant was chromatographed in solvent A for 30 h at 21°C. The UV-absorbing band at R_{UDP-GlcNAc} = 0.55 was cut out and washed in ethanol, and water was used to elute the nucleotide from the paper. The eluates were lyophilized, dissolved in water, and lyophilized again. Based on radioactivity, the final yield was 100 nmol (30,000 cpm) of nucleotide-X.

Nucleotide-X was initially characterized by descending paper chromatography in solvent B (15). A UV-absorbing spot migrating at R_{UDP-GlcNAc} = 0.2, a value expected for UDP-ManNAcUA (15), was observed. The UV-absorption spectrum of the nucleotide showed it to be a uridine nucleotide. To identify the sugar moiety, nucleotide-X was hydrolyzed in 1 N HCl for 25 min at 100°C and subsequently evaporated to dryness under vacuum over KOH pellets. The hydrolysate was dissolved in water and subjected to paper electrophoresis in solvent E (Fig. 2). Two ninhydrin-stainable amino sugars were observed. One of the amino sugars, migrating at position I, corresponds to the “free form” of 2-amino-2-deoxy-d-mannosaminuronic acid when compared to the standard. The second spot, at position III, moved to the same region as the positively charged 3,6-lactone of the d-mannosaminuronic acid standard. Furthermore, radioactivity was detectable only in the two ninhydrin-stained spots. These results identify the sugar moiety of nucleotide-X as being a hexosaminuronic acid or its acetylated form; however, solvent E does not differentiate mannosaminuronic acid from other hexosaminuronic acids. Consequently, the hydrolysate was subjected to paper electrophoresis in solvent F (Fig. 3). With the AgNO₃ stain, two spots were ob-

**Fig. 2. Identification of the sugar moiety of nucleotide-X by paper electrophoresis in solvent E (see the text).** (A) Hydrolysate of ECA containing free mannosaminuronic acid, its 3,6-lactone, and glucosamine. (B) Hydrolysate of ⁴⁻C-labeled nucleotide-X. (C) Glucosamine. (D) N-acetylglicosamine. If is an oligosaccharide containing III and IV (B). The compounds were detected by ninhydrin, except N-acetylglicosamine, which was detected by the procedure of Morgan and Elson as described by Neuberger and Marshall (12).
No detectable product was observed at UDP-GlcNAc concentrations of 0.02 mM and below. However, the amount of product made increased proportionately with the substrate concentrations at 0.1 mM and above. At UDP-GlcNAc concentrations of 0.45 mM and above, there was no appreciable increase in the amount of product.

We then measured the specific activity of this conversion reaction in extracts of various derivatives of S. typhimurium (Table 1, column A). In the his-rfb deletion mutants studied, all mutants that had deletions not extending beyond
FUNCTION OF rff GENES IN S. TYPHIMURIUM

TABLE 1. Enzymatic synthesis of UDP-ManNAcUA from UDP-GlcNAc in rff and rfe mutants of S. typhimurium

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genetic constitution</th>
<th>UDP-GlcNAc → UDP-ManNAcUA (A)</th>
<th>UDP-GlcNAc-2-epimerase (B)</th>
<th>UDP-ManNAc dehydrogenase (C)</th>
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<tbody>
<tr>
<td></td>
<td>rfb rfe rff</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>LT2</td>
<td>+ + +</td>
<td>25</td>
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<td>ND</td>
</tr>
<tr>
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<td>Δ650</td>
<td>14</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>his-515</td>
<td>Δ515</td>
<td>11</td>
<td>20</td>
<td>7.0</td>
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<tr>
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<td>Δ388</td>
<td>Trace</td>
<td>1.3</td>
<td>23</td>
</tr>
<tr>
<td>SH5824</td>
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<td>20</td>
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<td>&lt;3</td>
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</tr>
<tr>
<td>SH4892</td>
<td>4020 + 4215</td>
<td>11</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Units of activity expressed as nanomoles per milligram per hour. ND, Not determined.

3 Strains bearing his mutation numbers are deletion mutants in the his-rfb region of the chromosome, characterized earlier (13) and described in Fig. 1. Strains with extensive deletions ("his-399," "his-809," "his-386," and "his-519") have added rff mutations, as shown; nevertheless these strains were designated by their his deletion numbers in quotation marks, to facilitate comparison with earlier papers. SH4865 and SH4892 are a nearly isogenic pair, one containing a rfe mutation; for details see Lew, Nikaido, and P. H. Mäkelä, manuscript in preparation.

The genes for CDP-aborque biosynthesis (e.g., his-801, his-515) and were rff" and capable of synthesizing UDP-ManNAcUA. All deletion mutants, which had deletions extending beyond rffF gene (e.g., "his-399," "his-809," "his-519") and had accumulated secondary rff mutations (10), were found not capable of synthesizing UDP-ManNAcUA. When the rff" allele was reintroduced into the latter mutants by conjugation (10), there was a reappearance of the enzyme activity (see SH5803 and SH5805, Table 1). Finally, new rff mutants, obtained from these rff" rff strains by taking advantage of the fact that the former strains, but not the latter, are resistant to sodium dodecyl sulfate (10), have again lost most of the activity to convert UDP-GlcNAc into UDP-ManNAcUA (see SH5817 and SH5824, Table 1). Thus in all strains, there is a perfect correlation between the presence of rff" allele(s) and an ability to produce UDP-ManNAcUA.

The possibility that rff mutants might be producing an inhibitor of UDP-ManNAcUA synthesis was ruled out by mixed extract experiments. Thus the addition of crude enzyme extracts from various rff mutants defective in UDP-ManNAcUA synthesis did not inhibit the activity of an extract from a rff" strain (data not shown). Normal levels of enzymatic activity were found in an rfe mutant derived from LT2, as well as its isogenic rfe" recombinant (see SH4865 and SH4892, Table 1). Evidently, mutations at the rfe locus do not impair UDP-ManNAcUA synthesis. Additional evidence supporting this conclusion comes from similar results obtained with a Salmonella montevideo strain containing another rfe mutation (rfe-3623) (data not shown).

Synthesis of UDP-ManNAc from UDP-GlcNAc. Because the conversion of UDP-GlcNAc to UDP-ManNAcUA involves two enzymatic steps (4), the mutants described above could be defective in either one or both enzymes. Consequently, the UDP-GlcNAc epimerase was assayed by a modification of the method of Kawamura et al. (4) (see Materials and Methods). Under our conditions, the reaction was proportional to the amount of enzyme added, up to 300 μg (Fig. 6), and we routinely used 150 to 200 μg. It was proportional to the incubation time up to 30 min (Fig. 7). At initial UDP-GlcNAc concentrations of 0.4 mM and above, the ratio of UDP-ManNAc to UDP-GlcNAc after extensive incubation was about 1:10, in agreement with the equilibrium ratio determined by Kawamura et al. (4). Much larger proportions of UDP-ManNAc, however, were
produced when initial concentrations of UDP-GlcNAc were less than 25 μM; we have no explanations at present for this unexpected result. The assay of various mutant extracts (Table 1, column B) showed that one mutant, "his-809," lacked detectable epimerase activity, and "his-399" had only one-third the activity present in the wild type. All other strains tested had approximately 20 or more units of activity.

**Synthesis of UDP-ManNAcUA from UDP-ManNAc.** The second enzyme, UDP-ManNAc dehydrogenase, was assayed in various rff mutants (Table 1, column C). All of the rff mutants that had normal levels of UDP-GlcNAc 2-epimerase activity were found to be missing the dehydrogenase activity. Mutant "his-809" was the only rff mutant lacking activity in both enzymes, although "his-399" lacked dehydrogenase activity but contained a low level of 2-epimerase activity. When the rff+ allele was reintroduced into two rff mutants, the dehydrogenase activity was restored (see SH5803 and SH5805, Table 1). Finally, the rff mutants, derived from these rff+ rff- strains by selection to resistance to sodium dodecyl sulfate, were found lacking dehydrogenase activity (see SH5817 and SH5824, Table 1).

The dehydrogenase activity measured in rff+ strains was only one-third the activity of the conversion of UDP-GlcNAc to UDP-ManNAcUA in these same strains. The reason for this observation is that we are not measuring the dehydrogenase activity under the optimum conditions. The first step of the assay converts less than 10% of UDP-GlcNAc to UDP-ManNAc (4; also the preceding section) and may also result in significant degradation of these sugar nucleotides. Nevertheless, the dehydrogenase assay seems valid for the purpose of comparing nearly isogenic strains. No detectable dehydrogenase activity was observed in the absence of NAD+.

**DISCUSSION**

The data presented show that, in S. typhimurium, a constituent of ECA, ManNAcUA, is synthesized by the following reactions.

\[
\text{UDP-GlcNAc} \rightarrow \text{UDP-ManNAc (UDP-GlcNAc 2-epimerase)}
\]

\[
\text{UDP-ManNAc + NAD}^+ \rightarrow \text{UDP-ManNAcUA + reduced NAD} + \text{H}^+ \text{ (UDP-ManNAc dehydrogenase)}
\]

These reactions were previously demonstrated in E. coli by Ito and his co-workers (3, 4), and this work confirms their conclusion and extends it into S. typhimurium. More significantly, our work has shown that the loss of the dehydrogenase activity in rff mutants results in the inability to synthesize ECA, thus proving that at least this enzyme is absolutely necessary for the biosynthesis of ManNAcUA in intact cells. It seems especially important, in the case of this pathway, to establish its physiological significance through mutant studies, as mammalian UDP-GlcNAc 2-epimerase is known to release free ManNAc and UDP as reaction products (2, 16) in contrast to the E. coli and S. typhimurium
enzyme. In fact, since we could not detect any mutants singly defective in UDP-GlcNAc 2-epimerase among ECA− (rff) mutants, the absolute necessity for this enzyme has not yet been demonstrated, and one of the possible explanations for our results might be the presence of an alternative mechanism for the generation of UDP-ManNAc. In passing we note that the enzymatic activity level of UDP-ManNAcUA synthesis in rff− ECA+ strains is more than sufficient to synthesize the mannaminuronic acid present in ECA, which comprises 0.2% of the dry weight of Salmonella cells (8).

Our results showed that all of the six rff mutations studied resulted in the loss of the UDP-ManNAc dehydrogenase activity. It seems likely that the mutations occurred in the structural gene for the dehydrogenase, which we propose to call rffD. In one of the mutations (rff-4270), the 2-epimerase activity was also lost; this mutation could be either a polar one or a deletion. In another (rff-4271), the epimerase activity was only lowered. These results suggest that the structural gene of the 2-epimerase, which we propose to call rffE, is also located in the rff gene cluster, and that rffD is probably transcribed before rffE if the lowering of the epimerase activity in rffD271 is indeed due to the polarity effect.

Other gene clusters involved in ECA synthesis include rfb and rfe. The rfb cluster is not needed for the biosynthesis of UDP-ManNAcUA, as seen from the normal enzymatic activity in SH5803 (Table 1), which has a deletion including all the known genes of the rfb cluster. There is no evidence so far that the rfe cluster is needed for this purpose, as the two rfe mutants examined showed normal activity for UDP-ManNAcUA biosynthesis (Table 1 and Results). The possible functions of the rfb and rfe clusters in ECA biosynthesis will be discussed in a forthcoming paper (H. C. Lew, H. Nikaído, and P. H. Mäkelä, manuscript in preparation).

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LITERATURE CITED