Specific incorporation of glycine into bacterial lipopolysaccharide. Novel function of specific transfer ribonucleic acids

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Received April 26, 1991; Revised and Accepted September 20, 1991

ABSTRACT

It has been found that the bacterial endotoxins (lipopolysaccharides, LPSs) contain some amino acids and glycine is the most abundant amino acid in the polysaccharide core preparations of LPSs of gram-negative bacteria. Until now nothing was known about the mechanism of amino acid incorporation into the lipopolysaccharide core. We found that one out of three glycyl-tRNAs\(^{\text{Gly}}\) from \textit{Escherichia coli} is the donor of amino acid and is the substrate for a putative aminoacyl-tRNA:LPS transferase. We have isolated, purified this tRNA and determined its nucleotide sequence to be major \textit{E.coli} tRNA\(^{\text{Gly}}\). This tRNA\(^{\text{Gly}}\) (anticodon GCC) conserved the tRNA structural features. The assay for determination of the specific incorporation of glycine into the lipopolysaccharide was also invented and described.

INTRODUCTION

Prevention of gram-negative bacterial infections is still one of major medical problems. The conserved compounds of the bacterial membrane surface have been tested as the target for elaboration of the most effective vaccine. The search for a common epitope seems to be the best approach to find a vaccine of broad specificity. Such epitopes can be found in the outer membrane, which is composed of various proteins, phospholipids, lipoproteins and most notably, endotoxins. Chemically, the endotoxins are lipopolysaccharides (LPSs). The bacterial LPS possess two activities: the O-antigenic and endotoxic properties. The O-specificity is localized on the polysaccharide part which is built up of oligosaccharide repeating units. On the other side, for endotoxic activity a lipid part of LPS, called lipid A, is responsible.

Recently great interest was given to another part of bacterial lipopolysaccharide, namely the core oligosaccharide which spans O-antigen and lipid A and it shows rather conservative structure. Actually a high portion of the cores proved to be unsubstituted O-chains. Generally the core oligosaccharide is considered to be mediator of such biological activities as induction of interleukin-1 (1), binding LPS to hepatocytes (2), interaction with serum protein factor what leads to complement activation (3) and induction of antibodies against the common core epitopes (4). In addition to sugar component, several amino acids have been identified in the core preparations (5). However the function of those amino acids remain unknown. The alkali labile character of aminoacyl derivative component suggests its O-ester linkage. Many years ago Gentner and Berg have suggested the possibility of enzymatic formation of glycyl lipopolysaccharide in \textit{E. coli} from glycyl-tRNA\(^{\text{Gly}}\) (6). One can suppose, that identification of amino acid derivative of LPS could be very helpful to find specific target for preparation of general vaccine.

To understand this phenomenon we have undertaken studies on the mechanism of amino acid attachment to lipopolysaccharides. We showed unequivocally that incorporation of glycine to the LPS is mediated by one of the major \textit{E.coli} glycine-specific transfer ribonucleic acids for which we determined the nucleotide sequence. The acceptor properties of LPS for the amino acid residue from Gly-tRNA\(^{\text{Gly}}\) depends on the chemical structure of core oligosaccharide. We showed that the terminal galactose residue is the target for glycine.

We also found that the core oligosaccharide from strain C 600 of \textit{E.coli} is a good substrate for putative aminoacylation of tRNA:LPS transferase. In this paper we developed also a new assay for aminoacyl-LPS, which could be very useful in purification of the LPS-transferase. This project is now underway.

MATERIALS AND METHODS

Sources of lipopolysaccharides

\textit{Shigella sonnei} phase I standard strain 9773 (originally from the Dysentery Reference Laboratory, London), derived from the complete core spontaneous mutant phase II and its rough mutant \textit{ReP} were from the stock collection of the Institute of Immunology, Wrocław, Poland. \textit{Escherichia coli} strain K12 and
C 600 (PCM 2184) were obtained from the Microbiology Department of the University of Wroclaw, Poland. The bacteria were cultivated in liquid medium with aeration at 37°C (7). After 24 hours cells were harvested and dried with acetone for LPS extraction.

The lipopolysaccharides were prepared using phenol-water or phenol-chloroform-petroleum ether extraction exactly as described (7). Further purification was done using Sepharose 2B gel filtration (8). The lipopolysaccharide of Salmonella minnesota mutant strain Re 595 was purchased from Sigma (St Louis, USA).

Preparation of crude protein extract from Escherichia coli

20 ml of cells were grown to late logarithmic phase, washed and resuspended in 1.5 ml of 50 mM Tris-HCl pH 7.5, 1 mM EDTA. The soluble extract was prepared by sonication (4x10 s), centrifugation for 20 min at 12000×g and stored in 50% glycerol. It was used as the crude aminoacyl-tRNA synthetases preparation.

Escherichia coli crude tRNA was obtained from Sigma (St. Louis, USA) and chromatographed on benzoylated-DEAE cellulose (BD-cellulose), (9). The major glycine tRNA species were further purified on DEAE Sephadex A-50 column as described previously (9). The final purification was done with two dimensional gel electrophoresis (10). The analysis of nucleotide sequence was done by limited hydrolysis with formamide at 80°C, post-labeling with [γ-3P] ATP and T4 kinase and identification of 5' nucleotide was essentially as described previously (10).

The aminoacylation and the incorporation of glycine into lipopolysaccharide

The assay (11) in total volume of 0.05 ml contained 5 μmoles HEPES buffer pH 8.0, 0.2 μmole ATP (Na2), 0.3 μmole MgCl2, 0.1 μmole 2-mercaptoethanol, 3−5 μmole of KCl, 0.1 μmole of [14C]glycine, 32 μg of crude or specific tRNA, 1 μl of cell free extract with enzyme and 8 μl saturated solution of LPS. Incubation was carried out at 37°C for 1 hour. Then the mixture was cooled down and applied to a 15% SDS polycrylamide gel for electrophoresis (12, 13). When bromophenol blue reached the bottom of the gel, electrophoresis was finished and the gel stained with silver (14). The bands on the gel corresponding to LPS were cut off, put into scintillation mixture (Packard Filter Count) and radioactivity of amino acids counted.

RESULTS AND DISCUSSION

During structural analysis of the lipopolysaccharides (LPSs) from certain bacteria we surprisingly found that the LPSs contain some amino acids, of which glycine occurs in the highest quantities (Table 1), (16). The two types of the lipopolysaccharides were analyzed: one with and the other without O-substituted part (PS).

The lipid A and the high molecular mass polysaccharide fractions FI and FII from Shigella sonnei posses the majority of amino acids which derive from proteinaceous contaminants, usually present in trace amounts in the LPS preparations. However in the core FI, the amount of glycine is approximately one order of magnitude higher than other amino acids. The analysis of core FII showed presence of glycine only. One could ask the question if occurrence of this particular amino acid in the core oligosaccharide has some meaning e.g. whether it is specific for the bacteria, the LPS structure or to sugar residues? The crucial question in this respect seemed to be connected with mechanism of aminoacyl-LPS biosynthesis. To solve these problems we used several LPSs and their derivatives as the potential acceptors of glycine in the enzymatic transfer reaction. The analysis of [14C]glycyl-LPS formation was done on the SDS-polycrylamide gel. Different migration of Gly-tRNA Gly and Gly-LPS makes this technique very useful assay, even if the bands are not sharp enough. This is due however to chemical nature of LPS and their behavior on the polycrylamide gel. The band corresponding to the level of LPS (marked with arrow on lane 1, Figure 1), was cut off and analyzed in scintillation counter. Results of glycine incorporation to the LPS are shown in Figure 2. The data suggest unequivocally that the true substrate for putative transferase is just aminoacyl-tRNA e.g. glycyl-tRNA Gly. In this paper we concentrated ourselves on two important questions. First, which of glycyl-tRNAs Gly species of E.coli is responsible for this non-ribosomal specific transfer reaction activity and the second, what are the structural requirements of the lipopolysaccharides to fulfil this reaction.

Table 1. The percent composition of amino acids and other amino compounds in the Shigella sonnei two types lipopolysaccharides A and B and their fractions.

<table>
<thead>
<tr>
<th></th>
<th>GlcN</th>
<th>P-EtN</th>
<th>EtN</th>
<th>Asx</th>
<th>Glx</th>
<th>Ser</th>
<th>Gly</th>
<th>Arg</th>
<th>Thr</th>
<th>Alo</th>
<th>Tyr</th>
<th>Met</th>
<th>Val</th>
<th>Phe</th>
<th>Ile</th>
<th>Leu</th>
<th>Lys</th>
</tr>
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<tbody>
<tr>
<td>LPS FI</td>
<td>3.212</td>
<td>0.0816</td>
<td>0.158</td>
<td>0.058</td>
<td>0.103</td>
<td>0.056</td>
<td>0.094</td>
<td>0.089</td>
<td>—</td>
<td>0.007</td>
<td>0.028</td>
<td>0.025</td>
<td>0.028</td>
<td>0.002</td>
<td>0.004</td>
<td>0.009</td>
<td>—</td>
</tr>
<tr>
<td>Lipid A</td>
<td>27.30</td>
<td>0.7053</td>
<td>0.539</td>
<td>0.321</td>
<td>0.442</td>
<td>0.140</td>
<td>0.575</td>
<td>0.263</td>
<td>0.709</td>
<td>0.203</td>
<td>0.123</td>
<td>0.123</td>
<td>0.132</td>
<td>0.094</td>
<td>0.196</td>
<td>0.202</td>
<td>—</td>
</tr>
<tr>
<td>PS FI</td>
<td>0.881</td>
<td>0.143</td>
<td>0.221</td>
<td>0.074</td>
<td>0.021</td>
<td>0.069</td>
<td>0.074</td>
<td>—</td>
<td>0.049</td>
<td>—</td>
<td>0.083</td>
<td>0.062</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Core A</td>
<td>7.160</td>
<td>1.149</td>
<td>0.123</td>
<td>0.023</td>
<td>0.062</td>
<td>0.045</td>
<td>0.180</td>
<td>0.022</td>
<td>—</td>
<td>0.017</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.013</td>
<td>0.029</td>
</tr>
<tr>
<td>LPS FII</td>
<td>1.359</td>
<td>0.104</td>
<td>0.037</td>
<td>0.054</td>
<td>0.039</td>
<td>0.026</td>
<td>0.066</td>
<td>—</td>
<td>0.042</td>
<td>0.039</td>
<td>0.008</td>
<td>—</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.018</td>
<td>0.019</td>
</tr>
<tr>
<td>Lipid A FII</td>
<td>35.01</td>
<td>0.248</td>
<td>0.755</td>
<td>0.111</td>
<td>0.348</td>
<td>0.028</td>
<td>0.183</td>
<td>0.025</td>
<td>0.238</td>
<td>0.051</td>
<td>0.032</td>
<td>—</td>
<td>0.026</td>
<td>0.032</td>
<td>0.019</td>
<td>0.042</td>
<td>0.112</td>
</tr>
<tr>
<td>Core FII</td>
<td>2.175</td>
<td>0.100</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.095</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<td>—</td>
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</table>
Using benzoylated DEAE-cellulose (BD-cellulose) and DEAE-Sephadex A-50 column chromatography we isolated the three tRNA<sub>Gly</sub> subspecies from crude E.coli tRNA and purified them to homogeneity. Only one of them, the tRNA<sub>Gly</sub> (subspecies 3) showed glycine transfer activity (Figure 2). We determined its nucleotide sequence by post labeling method (10) and found it to be the major E.coli tRNA<sub>Gly</sub> (Figure 3). It contains anticodon GCC and only few modified nucleosides. The primary structure of this tRNA is identical to that already published of E.coli tRNA<sub>Gly</sub> (17). The differences in the nucleotide sequence between the tRNA<sub>Gly</sub> (the active isoacceptor in the transfer reaction) and two other tRNAs<sub>Gly</sub> isoacceptors (nonactive) are rather small and occur only in amino acid and dihydrouridine stems. To get information on the identity of glycyl-tRNA in the aminoacylation reaction of lipopolysaccharide we decided to analyze the activity of some tRNAs<sub>Gly</sub> mutants. The tRNAs<sub>Gly</sub> mutants were obtained by site directed mutagenesis (18). Here we have used two constructions based on E.coli tRNA<sub>Gly</sub>. In one of them, the anticodon loop and stem are the same as in Mycoplasma tRNA<sub>Gly</sub> while the rest of molecule is derived from E.coli tRNA<sub>Gly</sub>. The other construct contains the anticodon UCC in tRNA<sub>Gly</sub> structure where U32 was replaced by C32 (18). Our analysis showed that only one of these mutants can participate in Gly-LPS formation (data not shown). We analyzed and confirmed its primary structure by nucleotide sequencing (10). It is shown on Figure 4. The high homology between both tRNAs<sub>Gly</sub> active in this process (Figures 3 and 4) is clearly seen. However from the nucleotide sequence comparison of both tRNAs it is difficult to find tRNA identity elements for the LPS modification reaction, mainly because these tRNAs<sub>Gly</sub> species (Figures 3 and 4) are lacking unusual structural properties. This is in contrast to two glycine specific tRNAs from S.aureus which participate in peptidylglycan synthesis. Both of them are lacking the two conserved Gs in the dihydrouridine loop (22). Because tRNAs<sub>Gly</sub> participating in Gly-LPS formation have the normal

Figure 1. The assay for detection of the aminoacylated lipopolysaccharides on SDS gel. 15% SDS polyacrylamide gel electrophoretic analysis of aminoacylation of lipopolysaccharides (Sh. sonnet FII) from aminoacylated tRNA and the LPS. The gel was stained with silver (14). Lane 1 — assay mixture as in Materials and Methods containing both LPS and crude tRNA. Lane 2 — Assay mixture without tRNA. Lane 3 — Assay mixture without LPS. After electrophoresis the 5 mm pieces of the stained gel marked with arrow were cut off, dissolved in scintillation solution (Packard) and counted. The amount of radioactivity incorporated by different tRNA<sub>Gly</sub> is shown on Figure 2.

Figure 2. The level of [14C]glycine incorporation into the E.coli C 600 LPS using different glycine tRNA samples. (Abbreviations are as used in the text).

Figure 3. The primary structure of major glycine tRNA (anticodon GCC) from E.coli.

Figure 4. The nucleotide sequence of E.coli mutant tRNA<sub>Gly</sub> (anticodon UCC) (18).
Table 2. The chemical structures of the different cores of various LPSs and their ability to incorporate \( l^4 \)Cglycine. The galactose residues which are putative glycine binding sites are underlined. The activities of LPS were measured (cpm) as described in Materials and Methods.

<table>
<thead>
<tr>
<th>No</th>
<th>Source of LPS</th>
<th>Activity of LPS (cpm)</th>
<th>Structure of lipopolysaccharide</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>R S. minnesota</td>
<td>25</td>
<td>Kdo—lipid A</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>537 Hafnia</td>
<td>18649</td>
<td>unknown</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>R288 Sh. sonnei</td>
<td>37</td>
<td>unknown</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>2a Sh. flexneri</td>
<td>43</td>
<td>unknown</td>
<td></td>
</tr>
</tbody>
</table>

tRNA structure with all conserved elements one can conclude that in addition to their involvement in protein biosynthesis, they also participate in the transfer reaction. It is not known however what a regulation mechanism or structural requirements exist, which direct the particular tRNA for various functions. Until now, there is no answer for this question.

Another important problem, we would like to answer in this paper, is the structural properties of the core oligosaccharide of LPS itself which are necessary for binding aminoacyl residue. To prove it, we analyzed the chemical structure of a few LPS substrates and their derivatives with the aim to find the target for glycine residue (Table 2). It is interesting to notice, that all the sugar compounds which accept glycine, exclusively have the side chain with terminal galactose residue (underlined in Table 2). The lipopolysaccharides without the acceptor properties are lacking the galactose residue and have another tertiary structure (19). Therefore we suggest that terminal galactose is just target for the reaction of aminoacylation of the LPS.

It is well known that the enzymes called aminoacyl-tRNA transferases exist in cells (19). Those enzymes could be divided into the three groups according to the type of acceptor: aminoacyl-tRNA-protein transferases, aminoacyl-tRNA-phosphatidyl-glycerol transferases and finally aminoacyl-tRNA-N-acetyl-muramyl-pentapeptide transferases. The high specificity of those enzymes towards their acceptors leads us to assume that there could be another class of transferases which uses the lipopolysaccharide core oligosaccharide as the specific target molecule (aa-tRNA:LPS transferases). The experiments on purification of this enzyme are now in progress in our laboratory.

The biological functions of the glycine linked to the oligosaccharide core and the aminoacylated lipopolysaccharide are unknown. Little is also known on the function of aa-
tRNA:LPS transferases. Therefore attempts are made to define the biosynthesis pathway of aminoacyl-LPS. One can suppose that the glycyl residue in the core oligosaccharide forms immunological epitope and may in turn form a common structural domain for larger number of bacterial strains (20). Hence the exact understanding of the mode of glycine attachment to the core acceptor is so important. The specific acceptor structural requirements are necessary in order to understand the function of glycinated or, in general, the aminoacylated LPS core.

ACKNOWLEDGEMENTS

This work was supported by grants from Polish Academy of Sciences and from World Health Organization as part of its Program for Vaccine Development. Thanks are due to Miss M. Siatecka for help in preparation of aminoacyl-tRNA synthetase. Material published in this paper was presented at the 14th International tRNA Workshop, Rydzyna, Poland, 4–9 May 1991 (Abstract book p. 176) and at the 15th International Congress of Biochemistry, Jerusalem, Israel, August 4–8, 1991 (Abstract book p. 150).

REFERENCES