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Interaction of mycobacterial polymethylpolysaccharides with paranaric acid and palmitoyl-coenzyme A: Structural specificity and monomeric dissociation constants

(polysaccharide–lipid interaction/fluorescence/circular dichroism/tryptophan/fatty acid synthetase)

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ABSTRACT **The long-chain polyenoic fatty acids α- and β-paranaric acid form complexes with the 6-*O*-methylglucose polysaccharide from *Mycobacterium smegmatis* as demonstrated by an enhanced fluorescence emission of the paranaric acid. This interaction is eliminated by digestion of the methylglucose polysaccharide with α-amylase and glucoamylase, which removes four hexose units from the nonreducing end of the chain. Titration of the methylglucose polysaccharide with either paranaric acid isomer suggests formation of a 1:1 complex with a dissociation constant (K_d) of 0.4 μM. The fluorescence emission of this complex is quenched by palmitoyl-CoA, which indicates that the paranaric acid can be displaced by the acoyl-CoA, a conclusion confirmed by gel filtration. The presumed polysaccharide/palmitoyl-CoA complex has a K_d of about 0.1 μM. Acoyl-CoA derivatives with shorter fatty acid chains and free palmitic acid compete less effectively, indicating that they form weaker complexes with the polysaccharide. The methylmannose polysaccharides with 12 or 13 sugar units also complex paranaric acid strongly ($K_d \sim 0.4$ μM), whereas the isomer with 11 sugar units complexes weakly.**

The methylglucose polysaccharide has been coupled to L-tryptophan methyl ester. The fluorescence emission spectrum of the attached tryptophan group is shifted to a shorter wavelength relative to *N*-acetyl-L-tryptophan methyl ester, and this effect is enhanced in the corresponding derivative made with the amylase-digested polysaccharide. The circular dichroism spectrum of the polysaccharide–tryptophan derivative shows three bands with negative ellipticity, in the 270–300 nm region, not observed in the amylase-digested derivative. These results imply that the tryptophan is in a more structured environment in the former than in the latter derivative. α-Paranaric acid binds to the polysaccharide–tryptophan conjugate and shows an enhanced fluorescence emission with partial quenching of the tryptophan fluorescence emission, suggestive of Förster energy transfer from tryptophan to paranaric acid.

Mycobacteria contain unusual polysaccharides of intermediate size in which many of the sugar units are partially *O*-methylated, thus conferring on the molecules a slight hydrophobicity. One class is called the 6-*O*-methylglucose lipopolysaccharide (MeGLP) because some of the sugar units are also acylated (1–3), whereas the other is called the 3-*O*-methylmannose polysaccharide (MeMP) (4, 5). Both are composed of hexose units predominantly or exclusively in α(1 → 4) linkage, which confers on the molecules a proclivity to assume the helical conformation characteristic of amylose (6).

Evidence has accumulated that these polysaccharides are involved in the regulation of fatty acid synthesis in mycobacteria (7). In cell-free systems, they stimulate the incorporation of acetate and malonate into long-chain fatty acid (7), and they alter the chain length distribution of the fatty acid products (8). These effects are attributed, in part, to a sequestering of palmitoyl-CoA (9), an ability the polymethylpolysaccharides are known to possess (10). The primary mode of action may be the release of acoyl-CoA from the synthetase complex (11).

Both MeMP and MeGLP occur in several isomeric forms (5, 12), and it is possible that the different isomers interact preferentially with different acoyl-CoA derivatives. As a sensitive probe of these postulated interactions, we have used fluorescent polyenoic acids (13). In this report, we show that α- and β-paranaric acid form a 1:1 complex with the methylglucose polysaccharide and that they can be displaced from the complex by palmitoyl-CoA which in turn forms a 1:1 complex with the polysaccharide. Because these studies can be carried out at concentrations below the critical micellar concentration of the lipid derivatives, we have been able to evaluate monomeric dissociation constants for the interactions. The results also show that the interaction depends on the structural integrity of the polysaccharide. Related unmethylated glucans do not form complexes, whereas complexation by the methylmannose polysaccharides is dependent on their chain length.

EXPERIMENTAL PROCEDURE

Materials. Porcine α-amylase (type 1A) and L-tryptophan methyl ester HCl were from Sigma; glucoamylase was isolated from *Rhizopus javanicus* (14). Sephadex A-25, C-25, G-25, and G-75 (fine) came from Pharmacia; Bio-Gel P-2 and P-4 (400 mesh) were from Bio-Rad. P-L Biochemicals supplied the acoyl-CoA derivatives, and *N*-bromosuccinimide and dicyclohexylcarbodiimide were from Eastman. *N*-Acetyl-L-tryptophan methyl ester was prepared according to Sawyer (15). α-Paranaric acid (*cis,trans,trans,cis*-9,11,13,15-octadecatetraenoic acid) was a gift from Robert Simoni, Stanford University, and β-paranaric acid (the all-*trans* isomer) was purchased from Molecular Probes. Both compounds were recrystallized from petroleum ether (bp 40°–60°) and were dissolved in methanol just before use. Concentrations of both acids were determined from the ultraviolet absorption (16). *N,N*-Dimethylformamide was distilled from CaH_2 and stored over type 4A molecular sieve.

6-*O*-Methylglucose polysaccharide MeGP was obtained by deacylation of MeGLP. The α-amylase/glucoamylase-digested polysaccharide (AG-MeGP) was prepared as described (17). The MeMPs were purified according to Maitra and Ballou (5).

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Abbreviations: MeGLP, methylglucose lipopolysaccharide; MeMP, methylmannose polysaccharide; MeGP, methylglucose polysaccharide; AG-MGP, MeGP digested with amylase and glucoamylase; MeGP-Trp and AG-MeGP-Trp, the products formed by coupling MeGP and AG-MeGP to L-tryptophan methyl ester.

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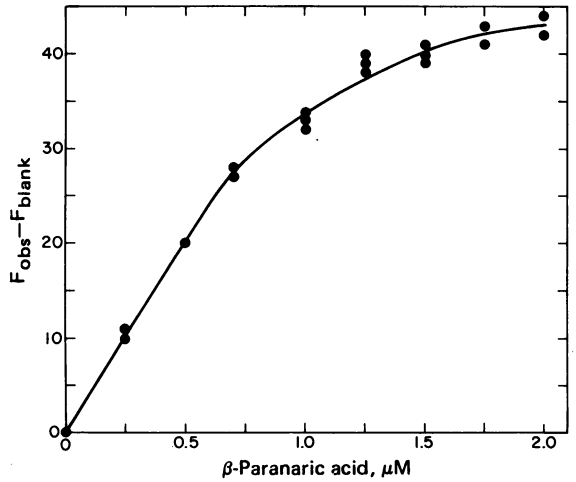

FIG. 1. Titration of MeGP with β-paranaric acid: Excitation at 328 nm and emission at 410 nm. The line is the curve obtained by a computer fit according to Eq. 1. Q_{AB} = 0.47 and K_d = 0.4 μM were obtained.

MeGP was coupled to L-tryptophan methyl ester as follows (18). MeGP (46 mg, 0.014 μmol) was dried over P_2O_5 under reduced pressure; 1 ml of 0.5 M L-tryptophan methyl ester HCl in dry dimethylformamide was added to the dry MeGP, followed by 1 ml of dry dimethylformamide containing 0.2 M dicyclohexylcarbodiimide. The reaction was stirred in the dark at 25° for 3 hr; then water was added to precipitate the dicyclohexylurea which was removed by centrifugation. The supernatant liquid was passed through a Millipore filter and concentrated to dryness under reduced pressure. The product was dissolved in water, and the solution was adjusted to pH 4 with dilute acetic acid and applied to a Sephadex C-25 (Na^+) column (2.5 × 12 cm) which then was eluted with water. The fractions that contained carbohydrate and showed absorbance at 280 nm were combined, lyophilized, and fractionated further on a Sephadex G-25 column by elution with water. Appropriate fractions were combined, lyophilized, and then fractionated on a Sephadex A-25 (HCO_3^-) column (1 × 15 cm) by elution with water. The neutral fractions were combined and lyophilized to yield a white fluffy powder. AG-MeGP was coupled to L-tryptophan methyl ester in the same fashion. Yields of MeGP-Trp and AG-MeGP-Trp were 60%–70%. Both showed a tryptophan-to-polysaccharide ratio of 1, based on *N*-bromosuccinimide oxidation of tryptophan (19) and total carbohydrate analysis by the phenol/sulfuric acid method (20) with D-glucose as the standard.

Methods. Ultraviolet spectra were recorded on a Cary 15 spectrophotometer and circular dichroism spectra, on a Cary 60 spectropolarimeter standardized with *d*-10-camphorsulfonic acid. Molecular ellipticities were calculated from the averages of three measurements (21), with a mean residue molecular weight for L-tryptophan amide methyl ester of 218.

Fluorescence spectra and measurements were recorded on a Spex Fluorolog fluorimeter. The binding of paranaric acids to polysaccharides was analyzed in terms of the equilibrium

polysaccharide + paranaric acid
⇄ polysaccharide–paranaric acid.

Dissociation constants and fluorescence enhancement factors for the complexes were determined by computer fit of the data according to the equation

$$F_{obs} = Q_{AB}\left\{\frac{K_d + A_T + B_T - |\sqrt{(K_d + A_T + B_T)^2 - 4A_TB_T}|}{2}\right\} - F_{blank} \quad [1]$$

Table 1. Interaction of polysaccharides and paranaric acid

Polysaccharide	Dissociation constants, μM* α-Paranaric acid	β-Paranaric acid
MeGP	0.39	0.40
AG-MeGP	†	†
MeGP-Trp	0.40	0.40
AG-MeGP-Trp	†	†
MeMP-II	0.38	‡
MeMP-III	0.40	‡
MeMP-IV	§	§
α1 → 4-Gluconononaose	†	†
α1 → 4-Glucohexadecanaose	†	†
α-Cyclodextrin	†	†

* Determined from computer fit of the observed fluorescence data of 0.5 μM polysaccharide solutions, titrated to 1 μM paranaric acid.
† No enhancement of paranaric acid fluorescence was observed.
‡ Not determined.
§ Slight evidence of interaction.

in which F_{obs} is the observed fluorescence of the polysaccharide–paranaric acid complex, F_{blank} is the fluorescence of paranaric acid alone, K_d is the dissociation constant of the complex, A_T is the total concentration of polysaccharide, B_T is the total concentration of paranaric acid, and Q_{AB} is the fluorescence enhancement factor of the complex.

Fluorescence titrations were done by adding aliquots of a stock solution of paranaric acid in methanol to a fixed concentration of polysaccharide in 0.01 M phosphate buffer, pH 7, by using a 1-μl syringe. In all experiments, time was allowed for mixing and equilibration. The volume change due to addition of the paranaric acid solution was less than 1%. Fluorescence emission of paranaric acid was recorded at 410 nm by excitation at 328 nm, whereas that of the tryptophanyl residue of MeGP-Trp and AG-MeGP-Trp was recorded at 344 and 342 nm, respectively, by excitation at 260 nm.

Complex formation was measured by gel filtration on a Sephadex G-75 column (0.3 × 30 cm) with elution with 0.01 M phosphate buffer, pH 7.0. Samples were prepared by adding the lipid in methanol to a test tube, removing the methanol by evaporation, and then adding the polysaccharide dissolved in buffer. The lipid was dispersed by sonication. The sample was applied to the column in a volume of 8 μl, and 120-μl fractions were collected. Each component was 250 μM when added to the column, and each was eluted in three fractions, so that at elution the concentrations were about 6 μM. The MeGP-Trp peak was detected by fluorescence at 344 nm after excitation at 292 nm, whereas the paranaric acid was detected by its fluorescence at 410 nm after excitation at 320 nm. To enhance the fluorescence of free paranaric acid, the column effluent was diluted with 2 ml of methanol.

RESULTS

Binding of Paranaric Acids to Polymethylpolysaccharide Derivatives. The fluorescence emission of paranaric acid, which is low in water, is enhanced at least 12-fold in the presence of polymethylpolysaccharides. Titration of MeGP or MeGP-Trp with β-paranaric acid gave a saturation curve (Fig. 1) that, when analyzed by a Scatchard plot (22), gave a polysaccharide-to-paranaric acid ratio of 0.7–1.2 mol/mol and a K_d of about 1 μM. By computer fitting, assuming a freely reversible 1:1 complex, similar data gave a K_d of 0.4 μM (Table 1). No

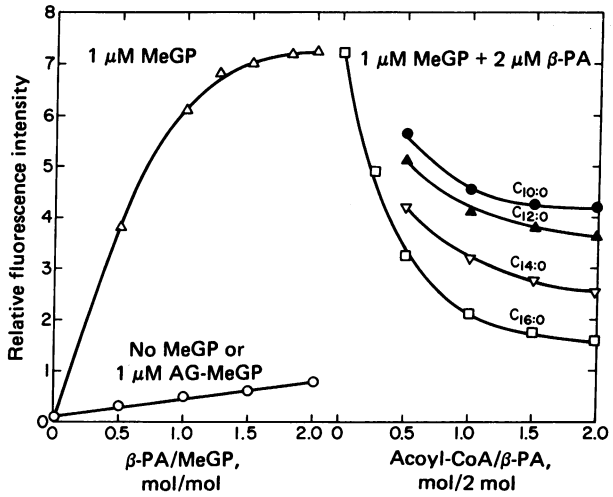

FIG. 2. Titration of MeGP with β-paranaric acid (β-PA) and displacement of β-paranaric acid by acoyl-CoA derivatives. In the *left-hand curve*, 1 μM MeGP was titrated to 2 μM β-paranaric acid. In the *right-hand curves*, a solution containing 1 μM MeGP and 2 μM β-paranaric acid was titrated to various acoyl-CoAs at 1 μM. No enhancement of the fluorescence emission was observed with AG-MeGP.

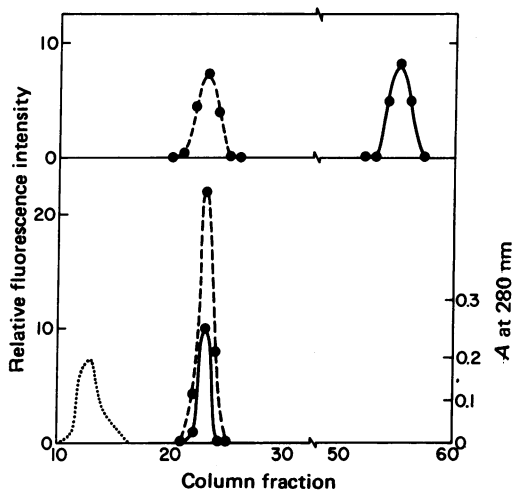

FIG. 3. Demonstration, by gel filtration (Sephadex G-75), of the displacement of paranaric acid by palmitoyl-CoA. —, Fluorescence emission of paranaric acid; - - -, fluorescence emission of MeGP-Trp; . . . . ., A_{280} of blue dextran. MeGP alone is eluted in fraction 23 and β-paranaric acid alone is eluted in fraction 56. The void volume is at fraction 13. (*Lower*) Elution of MeGP/paranaric acid mixture. The elution of blue dextran 2000, to determine the void volume, is also shown. (*Upper*) Elution of MeGP/paranaric acid/palmitoyl-CoA mixture. The decreased intensity of the fluorescence emission of MeGP-Trp in the presence of palmitoyl-CoA suggests that the lipid forms a complex that quenches the fluorescence. We confirmed this in a titration of MeGP-Trp by palmitoyl-CoA (data not shown).

evidence for complex formation was observed with AG-MeGP or unmethylated glucans under these conditions. MeMP-II and MeMP-III complexed paranaric acid as well as did MeGP, but MeMP-IV interacted poorly. At 100 μM concentrations, AG-MeGP as well as MeMP-IV showed evidence of weak interaction with 1 μM paranaric acid (data not shown).

Competition by Acoyl-CoA Derivatives for Polysaccharide–Paranaric Acid Binding. Titration of the MeGP–paranaric acid complex with palmitoyl-CoA led to a progressive quenching of the fluorescence emission (Fig. 2) that was consistent with the displacement of the paranaric acid by the acoyl-CoA. This interpretation is supported by the gel filtration study summarized in Fig. 3. MeGP and β-paranaric acid passed through a Sephadex G-75 column in coincident peaks; but, after addition of palmitoyl-CoA, the polysaccharide and paranaric acid eluted in different positions. That palmitoyl-CoA and polysaccharide form a complex under these conditions was confirmed by the quenching of the tryptophan fluorescence emission of MeGP-Trp.

These results are consistent with our conclusion that the polysaccharide and palmitoyl-CoA form a tighter complex than do the polysaccharide and paranaric acid. The curve in Fig. 2 shows that the fluorescence emission of the MeGP–paranaric acid complex is decreased 50% when the concentration of palmitoyl-CoA is 0.5 μM and that of β-paranaric acid is 2.0 μM, suggesting that the MeGP–palmitoyl-CoA complex has a K_d of 0.1 μM or less. Other acoyl-CoA derivatives were less effective competitors (Fig. 2), and free palmitic acid was about as good as myristoyl-CoA (data not shown).

Interaction of Tryptophanyl-Polysaccharides with Paranaric Acid. The carboxyl group of MeGP was coupled in good yield to the amino N of L-tryptophan methyl ester by the carbodiimide procedure (18). The product, MeGP-Trp, showed a shift in its emission spectrum (Fig. 4) that is consistent with the tryptophan group occupying a less polar environment than when free in solution. The circular dichroism spectrum (Fig. 5) showed three bands with negative ellipticity indicative of a structured or restricted tryptophan residue (23).

The absorption and emission spectra of tryptophan and paranaric acid are such that they can be excited independently and their fluorescence emission observed (Fig. 6). The paranaric acid absorption and tryptophan emission spectra also overlap slightly. The intrinsic fluorescence of MeGP-Trp was about 15% greater than that of Ag-MeGP-Trp. Titration of MeGP-Trp with α-paranaric acid showed almost a 50% enhancement of the paranaric acid fluorescence emission over that observed with MeGP itself, whereas there was no evidence of an interaction of paranaric acid with AG-MeGP-Trp (Fig. 7). Conversely, the fluorescence emission of the tryptophan residue of MeGP-Trp was quenched by the addition of α-paranaric acid, and the value approached that of the intrinsic fluorescence of uncomplexed AG-MeGP-Trp.

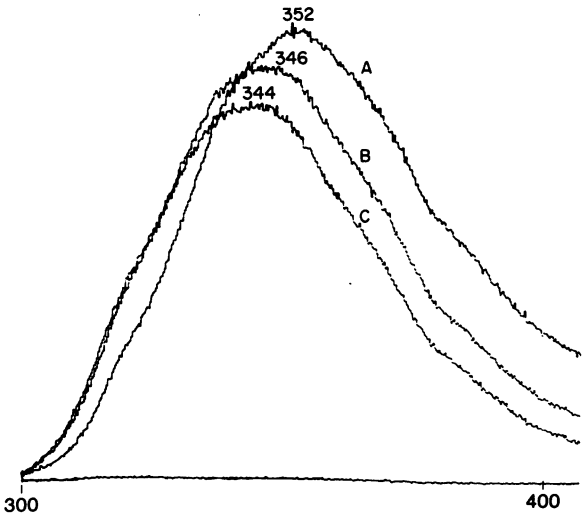

FIG. 4. Fluorescence emission spectra of the tryptophanyl group. Excitation was at 260 nm and the emission of *N*-acetyltryptophan methyl ester (A), MeGP-Trp (B), and AG-MeGP-Trp (C) were recorded. The maxima show a progressive shift to shorter wavelengths.

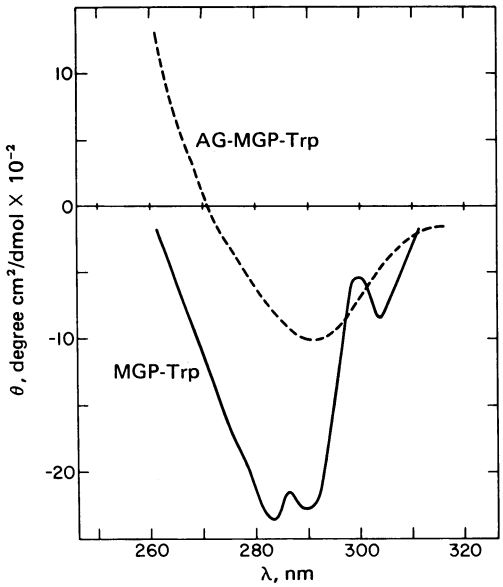

FIG. 5. Circular dichroism spectra of polysaccharide–tryptophan derivatives. The complex spectrum of MeGP-Trp (solid line) was not observed with AG-MeGP-Trp (dashed line).

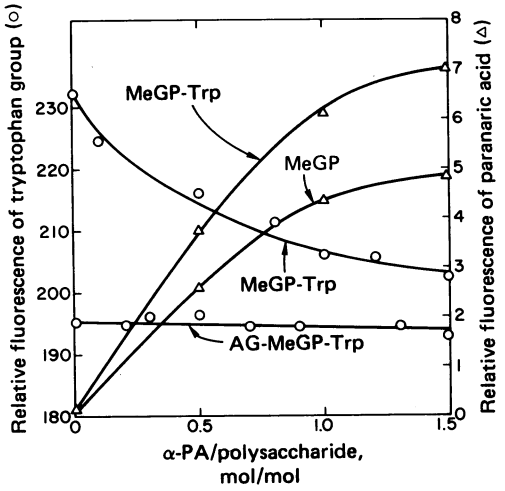

FIG. 7. Titration of MeGP derivatives with α-paranaric acid (α-PA), showing the enhancement of the paranaric acid fluorescence emission by the MeGP-Trp derivative compared to MeGP, the quenching of the tryptophan fluorescence emission in MeGP-Trp by paranaric acid, and the absence of an effect on the tryptophan fluorescence emission of AG-MeGP-Trp by paranaric acid.

DISCUSSION

Our objective was to design a sensitive and quantitative assay for the interaction of mycobacterial polymethylpolysaccharides with lipid derivatives below the critical micellar concentration, thus allowing the evaluation of monomeric dissociation constants and the recognition of subtle differences in specificity related to structure or conformation. The enhancement of the fluorescence emission that occurs when paranaric acid is added to a solution of MeGP has been successfully exploited for this purpose.

MeGP and β-paranaric acid form a 1:1 complex with an apparent K_d of 0.4 μM. β-Paranaric acid is displaced from the complex by palmitoyl-CoA to yield a 1:1 complex between polysaccharide and acoyl-CoA. Our results suggest an apparent K_d of about 0.1 μM for this reaction. Other acoyl-CoA derivatives with shorter fatty acid chains form weaker complexes, whereas palmitic acid binds with a constant similar to that of myristoyl-CoA. Although the shape of the polyenoic acid could be important for the binding, we find that the all-*trans* β-paranaric acid has about the same K_d for MeGP as α-paranaric acid, the *cis,trans,trans,cis* isomer.

Previous studies (10) of complex formation between polymethylpolysaccharides and acoyl-CoA derivatives were done at concentrations far above the critical micellar concentrations of the lipids, so that the measurements reflect interactions between polysaccharide and lipid micelles. Machida and Bloch (10) cited an apparent K_d of 160 μM for the MeGLP–palmitoyl-CoA complex, much higher than the value we find for the monomeric interaction. In spite of this, the relative strengths of the interactions they reported parallel those we observed. Perhaps more important than the evaluation of dissociation constants, we find a specific structural requirement for this interaction not previously apparent in the enzymatic system (10). The modification of MeGP by amylase digestion, which removes four hexose units from the nonreducing end of the chain to yield AG-MeGP, eliminates the interaction with paranaric acid.

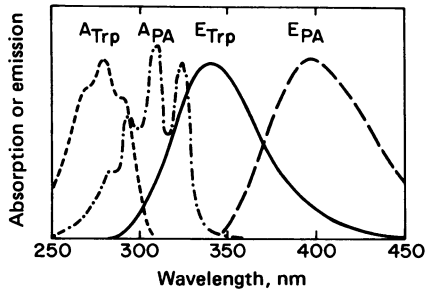

FIG. 6. Absorption and emission spectra of tryptophan and α-paranaric acid. Only the general shapes of the curves are shown, not the relative intensities. A_{Trp} and A_{PA}, absorption spectra; E_{Trp} and E_{PA}, fluorescence emission spectra.

To investigate the secondary structure of MeGP that might explain its ability to complex paranaric acid, we coupled MeGP through it glyceric acid residue (17) at the "reducing end" to the amino N of L-tryptophan methyl ester. In this derivative, MeGP-Trp, the fluorescence emission of the tryptophan group was shifted to a shorter wavelength, relative to *N*-acetyl-L-tryptophan methyl ester, as expected if the aromatic ring were held in a less polar environment. This shift was enhanced in AG-MeGP-Trp, although the intrinsic fluorescence emission was reduced. We conclude that the tryptophan group is folded into a nonpolar domain of MeGP and that removal of the four hexoses from the other end of the chain slightly enhances this nonpolar interaction.

The relationship between the paranaric acid binding site and the site occupied by the tryptophan group was investigated by titrating MeGP-Trp with the polyenoic acid. The paranaric acid fluorescence emission was enhanced and the fluorescence emission of the tryptophan group was quenched, which suggests that the two probes approach each other closely enough for energy transfer to occur (24) but that they do not compete for the same site. In agreement with these conclusions, the structural requirements are different for binding paranaric acid and for the blue shift in the tryptophan fluorescence emission. Thus, although amylase digestion of MeGP enhances the blue shift in the tryptophan fluorescence, it eliminates complexation of the paranaric acid. Apparently, the unmethylated hexoses at the nonreducing end of the polysaccharide chain help to stabilize a secondary structure that is essential for paranaric acid binding and that also affects the rotational freedom of the

tryptophan residue, but this conformation is not significantly involved in determining the hydrophobic environment of the tryptophan group.

Alternative models to rationalize these results depend on whether the lipid associates with the methyl groups on the surface of the polysaccharide or is included within the hydrophobic space of a helical chain. If the former, it is possible that the spacing of the methyl groups might be altered by a change in polysaccharide conformation. If the latter, one can postulate either that the cavity in the helix becomes too small for the fatty acid to enter or that it becomes too short for a stable complex to form. MeGP has predominantly an $\alpha 1 \rightarrow 4$-D-glucopyranose structure analogous to amylose, and it should have a similar tendency to assume a helical conformation. It is well documented that the coiled amylose chain contains a nonpolar space into which aliphatic alcohols and other lipid substances may intercalate (25). In fact, amylose binds palmitic acid tightly enough to displace iodine from the amylose–iodine complex. By analogy, we expect that MeGP would have two nonpolar domains—that resulting from the coiled conformation and that associated with the sequence of 10 methylated sugar units. Our results suggest that the covalently linked tryptophan group is a probe for one domain and that the complexation with paranaric acid involves the other domain.

Assuming that MeGP is a tightly coiled helix, it would have the dimensions of a cylinder about 15 Å in diameter and 30 Å long. If the tryptophan group is held at one end of the cylinder and the paranaric acid is inserted in the hydrophobic interior of the coil at the other end, the two probes could be as close as 5–10 Å, depending on how far into the coil the acid penetrates. If the coil were loose, the probes might be as far apart as 20–30 Å. Such an extended conformation might give the tryptophan group more rotational freedom than the circular dichroism spectrum suggests it has, unless the tryptophan is folded back upon the chain. In fact, the circular dichroism spectrum of AG-MeGP-Trp is consistent with the tryptophan group becoming less restricted on removal of the tetrasaccharide unit, as might occur if it was extended away from the chain concomitant with a tightening of the coil. Such a conformational change, however, would not be expected to give the blue shift in the fluorescence emission that is observed in converting MeGP-Trp to AG-MeGP-Trp.

Although, intuitively, the role of the methyl groups in the polysaccharide would seem to be directly to enhance the interaction with lipids, this supposition is inconsistent with the decreased paranaric acid binding on removal of the unmethylated tetrasaccharide unit. Such a change should increase the overall hydrophobicity of the molecule. If the effect of this structural modification, however, were to change the conformation of the polysaccharide into a more tightly coiled helix, the inclusion of the fatty acid might no longer be possible. The factors controlling a conformational change of this type could be a delicate balance between the organized water structure around the methyl groups and the hydrogen bonding of the sugar hydroxyl groups to solvent in an extended chain, versus the intramolecular hydrogen bonds and van der Waals interactions that would be possible in the tightly coiled helix. Alternatively, it is still possible that removal of the tetrasaccharide unit from MeGP shortens the coiled section of the polysaccharide just enough that the paranaric acid is unable to form a stable inclusion complex. In fact, this possibility is enhanced by our observation that paranaric acid forms complexes with MeMPs containing 12 or 13 hexose units, whereas complexation is much weaker in the isomer with only 11 sugar units.

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