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Structure of the *Escherichia coli* K2 Capsular Antigen. Stereochemical Configuration of the Glycerophosphate and Distribution of Galactopyranosyl and Galactofuranosyl Residues†

Werner Fischer,* M. Alexander Schmidt, Barbara Jann, and Klaus Jann

ABSTRACT: The *Escherichia coli* K2 capsular antigen is known to be composed of α-D-galactopyranosyl(1–2)glycerophosphate and α-D-galactofuranosyl(1–2)glycerophosphate units which are connected by phosphodiester bonds to C-4 of the galactopyranosyl and C-5 or C-6 of the galactofuranosyl moieties. In the present study the glycerophosphates were released by two different procedures and shown to have the *sn*-glycero-3-phosphate stereochemical configuration. In the first, the chain was fragmented by Smith degradation to glycerophosphothreitol from which the glycerophosphate was released by alkali hydrolysis. The structure-dependent low recovery of α-glycerophosphate (<10%) initiated the development of another degradative sequence which consisted of periodate oxidation, β elimination, hydrazinolysis, and alkaline treatment. This way, approximately 90% of the glycerophosphate was released as *sn*-glycero-3-phosphate. β elimination revealed in addition that most of the galactofuranosyl residues carry the phosphodiester bond at position 5. Separation by gel permeation chromatography and analysis of the fragments obtained by β elimination showed that pyranosidic and furanosidic galactosyl residues alternate in the same chain and suggested the sequences Gal*f*(*p*)GroP–(Gal*p*GroP)$_n$–Gal*f*– and –Gal*f*GroP–(Gal*p*GroP)$_n$–Gal*f*–, where *n* is 6, 4, and 3, respectively.

The occurrence of teichoic and lipoteichoic acids appeared to be confined to Gram-positive bacteria [for reviews, see Knox & Wicken (1973) and Lambert et al. (1977)] until recent investigations into the capsular antigen of *Escherichia coli* K2 and K62 revealed a poly(galactosyl(α1–2)glycero-1(3)-phosphate) structure (Jann et al., 1980; Jann & Schmidt, 1980) which was closely related to the poly(glycosylglycerophosphate)-containing types of teichoic (Burger & Glaser, 1966; Archibald & Coapes, 1971) and lipoteichoic acids (LTA)[1] (Koch & Fischer, 1978). In the previous work on these capsular antigens, the stereochemical configuration of the glycerophosphate residues was not investigated, and the arrangement of the pyranosidic and furanosidic galactosyl residues which were observed in both polymers remained open. Here we report on these two structural features of K2 antigen.

Materials and Methods

Materials. Escherichia coli K2 capsular antigen was prepared as previously described (Jann et al., 1980). Lipoteichoic acid from *Streptococcus lactis* Kiel 42172 stemmed from earlier work (Koch & Fischer, 1978). Cyclic 1(3),2-glycerophosphate was prepared according to the procedure of Ukita et al. (1955), erythritol phosphate by reduction of D-erythrose 4-phosphate with $NaBH_4$, and di-α,α′-glycerol phosphate by deacylation of phosphatidylglycerol (Fischer et al., 1973).

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[1] Abbreviations: Gal*p*, α-D-galactopyranosyl; Gal*f*, α-D-galactofuranosyl; GroP, *sn*-glycero-3-phosphate residue; LTA, lipoteichoic acid.

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Analytical Procedures. D-Galactose, glycerol, *sn*-glycero-3-phosphate, phosphate, and periodate were quantitated by established procedures [for the original references to these methods, see Fischer et al. (1973) and Nakano & Fischer (1978)]. Glycerophosphate and phosphomonoester were measured after hydrolysis with acid phosphomonoesterase (2.5 units/mL 0.05 M citrate buffer of pH 5.5, 37 °C, 1 h) as glycerol and inorganic phosphate, respectively. α-Glycerophosphate and β-glycerophosphate were quantitated after oxidation with $NaIO_4$ and subsequent hydrazinolysis (LeCocq & Ballou, 1964) as inorganic phosphate and glycerol, respectively. Polyols and polyol phosphates were trimethylsilylated and analyzed by gas–liquid chromatography essentially as in previous work (Fischer et al., 1973). Separation was performed on 0.3% OV 1 on Gaschrom Q (100/120 mesh) in a glass column (0.3 × 100 cm); the oven temperature was initially kept at 120 °C for 2 min and then raised by 6 °C/min.

Smith Degradation and Subsequent Alkali Hydrolysis. The previously described procedure (Koch & Fischer, 1978) was modified as follows: To avoid β elimination (see Results and Interpretation) oxidation with $NaIO_4$ (0.1 M) was done in 0.1 M sodium acetate buffer, pH 5.0, at 4 °C for the minimum time required for complete oxidation. This was determined by measuring the periodate consumption and testing hydrolyzed samples (1 M HCl, 100 °C, 2 h) with D-galactose dehydrogenase.

For separation of glycerophosphothreitol from its hydrolysis products, the acid hydrolysate (see Results and Interpretation) was neutralized with 0.1 M triethylammonium carbonate, pH 7.8, diluted with water, and applied to a column of DEAE-Sephacel (0.6 × 30 cm) preequilibrated in the same buffer and washed with water before use. Glycerol was eluted with water (30 mL), phosphodiester, and phosphomonoester with 20 and 40 mM triethylammonium carbonate, pH 7.8 (30 mL each), respectively.

Polyols and polyol monophosphates obtained on alkali hydrolysis were separated on the same column.

Sequential Degradation by $NaIO_4$ Oxidation, β Elimination, Hydrazinolysis, and Alkaline Treatment. Periodate oxidation was done as described above. Immediately after the addition of ethylene glycol, the pH was adjusted to 7 with $NaHCO_3$, an equal volume of freshly prepared 0.2 M triethylammonium carbonate, pH 10.5, was added, and the mixture was incubated at 25 °C. Phosphodiester cleavage was followed by measuring phosphomonoester and total phosphate. When β elimination was finished, the pH was adjusted to 5 with acetic acid (1 M), and a freshly prepared solution of 1,1-dimethylhydrazine (previously adjusted to pH 5 with acetic acid) was added to give a final concentration of 0.25 M and a 20-fold molar excess over $NaIO_3$. After standing at 37 °C for 24 h, the mixture was several times extracted with chloroform, then diluted 10-fold with water, and applied to a column (0.6 × 30 cm) of DEAE-Sephacel which was preequilibrated with 0.1 M triethylammonium carbonate buffer, pH 8.5, and washed with water before use. On elution with a linear gradient of the same buffer (0–0.3 M), a single phosphorus-containing peak emerged at a buffer concentration of 0.08 M. From the combined fractions, buffer was removed by several evaporations with water in vacuo. The products were analyzed as described under Results and Interpretation. Final alkaline treatment was done in 0.1 M NaOH at 100 °C for 2 h. Prior to analysis, the hydrolysate was neutralized with acetic acid.

Dependence of β Elimination on pH. UMP, disodium salt, was oxidized in unbuffered 0.1 M $NaIO_4$ solution (4-fold molar excess) at 4 °C for 1 h. Excess $NaIO_4$ was reduced with ethylene glycol. Buffer solutions for β elimination contained succinic acid (pH 5.5), 2-(*N*-morpholino)ethanesulfonic acid (pH 6.5), *N*-(2-hydroxymethyl)piperazine-*N'*-ethanesulfonic acid (pH 7.5), glycylglycine (pH 8.5), and triethylamine (pH 10.5), 0.10 M (final concentration). The elimination was followed at 22 °C by measuring inorganic phosphate and total phosphorus. Good first-order kinetics were observed at all pH values.

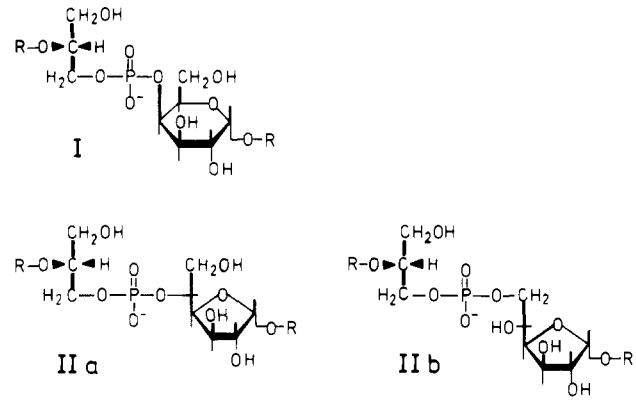

FIGURE 1: Repeating units of the *Escherichia coli* K2 antigen (Jann et al., 1980). About one-third of the D-galactosyl residues was shown to be in the furanosidic form, but it was not differentiated between IIa and IIb.

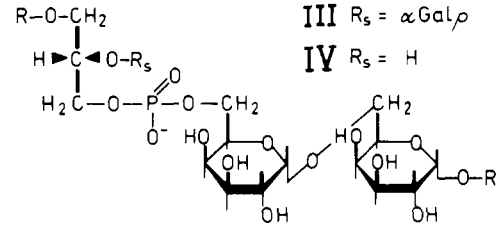

FIGURE 2: Repeating unit of the lipoteichoic acid (LTA) from *Streptococcus lactis* Kiel 42172 (Koch & Fischer, 1978). The glycerophosphate residues were shown to have the *sn*-glycero-1-phosphate stereochemical configuration. IV was prepared from III by treatment with α-galactosidase.

Results and Interpretation

Release of Glycerophosphates from K2 Antigen by Smith Degradation and Subsequent Alkali Hydrolysis. Figure 1 shows the repeating units of *E. coli* K2 capsular antigen (Jann et al., 1980). Figure 2 shows the repeating unit of a structurally related lipoteichoic acid (LTA) which served as reference compound in the present study. As with this LTA (Koch & Fischer, 1978), we released the glycerophosphates from K2 antigen for stereochemical analysis by Smith degradation and alkali hydrolysis of the resultant glycerophosphothreitol. Although alkali hydrolysis of phosphodiester-bound glycerols gives a mixture of α- and β-glycerophosphates (Baer & Kates, 1950), the released α isomer is appropriate for stereochemical configuration analysis since it retains the stereo configuration that it had in the parent compound (Brotherus et al., 1974; Fischer & Landgraf, 1975).

Oxidation under Controlled Conditions. After oxidation of K2 antigen in unbuffered $NaIO_4$ solution at ambient temperature for 18 h, chain fragmentation was observed on gel permeation chromatography (see below). A phosphomonoester/phosphate ratio of 0.23 ± 0.03 indicated phosphodiester cleavage since the starting material did not contain phosphomonoester. When under the same conditions di-α,α'-glycerophosphate and LTA were oxidized, the phosphodiester of the former remained intact, whereas LTA showed phos-

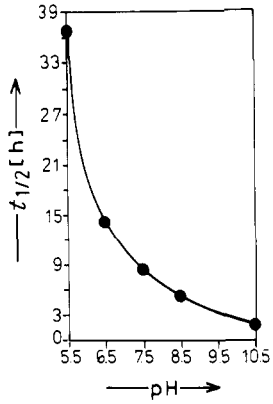

FIGURE 3: Dependence of β elimination on pH. Periodate-oxidized UMP was incubated at 22 °C in buffers of the indicated pH values, and the reaction was followed by measuring inorganic phosphate. Half-times ($t_{1/2}$) were taken from first-order velocity plots.

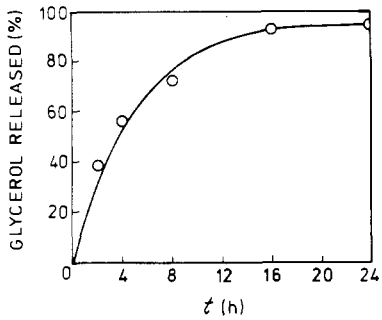

FIGURE 4: Time course of acid hydrolysis of acetal bonds. α-Kojibiosylglycerol was oxidized with $NaIO_4$ and the product reduced with $NaBH_4$ and purified as described previously (Fischer & Landgraf, 1975). The product was hydrolyzed in 0.05 M H_2SO_4 at 37 °C, and the reaction was followed by measuring the released glycerol. One hundred percent refers to the glycerol liberated by hydrolysis in 1 M HCl, 100 °C, for 2 h.

phodiester cleavage which, dependent on the reaction time (18–36 h), approached 30–50%. The structures of repeating units IIa and III (Figures 1 and 2) suggested that phosphodiester cleavage occurred by β elimination. As β elimination was commonly carried out at pH 10.5 (Brown et al., 1955; Archibald & Stafford, 1972), we tested its dependence on pH (Figure 3). As expected, the reaction rate slowed down with decreasing pH but was still measurable at pH 5.5.

For prevention of degradation, K2 antigen and LTA were oxidized in 0.1 M sodium acetate buffer, pH 5, at 4 °C for 2 and 16 h, respectively, by which times the oxidation was complete and phosphodiester cleavage negligible.

Smith Degradation. The oxidized K2 antigen was reduced with $NaBH_4$, and the product, after being desalted, was subjected to mild acid hydrolysis (50 mM H_2SO_4, 37 °C, 36 h). The phosphodiester proved to be particularily labile, with 42% being hydrolyzed as compared to 12% in the case of the LTA and di-α,α'-glycerol phosphate (Koch & Fischer, 1978). Shortening the period of hydrolysis to 18 h, the minimum time required for the hydrolysis of acetal bonds (Figure 4), still resulted in a phosphodiester cleavage of 26%. The molar ratio of free glycerol to phosphomonoester was 0.78, which, in accordance with previous observations (Jann et al., 1980), indicated that the threitol moiety favored cyclization of the phosphate group and by that enhanced phosphodiester cleavage.

Release of Glycerophosphate and Stereochemical Analysis. The remaining glycerophosphothreitol was purified by chromatography on DEAE-Sephacel and subjected to alkaline hydrolysis (0.1 M NaOH, 100 °C, 16 h). Like on acid hydrolysis, the molar ratio of free glycerol to phosphomonoester was between 0.78 and 0.85.

Table I: Analyses of the Glycerophosphates Released from Glycerophosphothreitol by Alkali Hydrolysis

compd	molar ratios to phosphorus[a]	
total glycerophosphate[b]	0.22	0.15
β-glycerophosphate[c]	ND	0.09
sn-glycero-3-phosphate[d]	0.10	0.07

[a] Values of two separate degradations of K2 antigen. [b] Measured as glycerol after hydrolysis with phosphomonoesterase. [c] Measured as glycerophosphate after periodate oxidation. [d] Measured with *sn*-glycero-3-phosphate dehydrogenase.

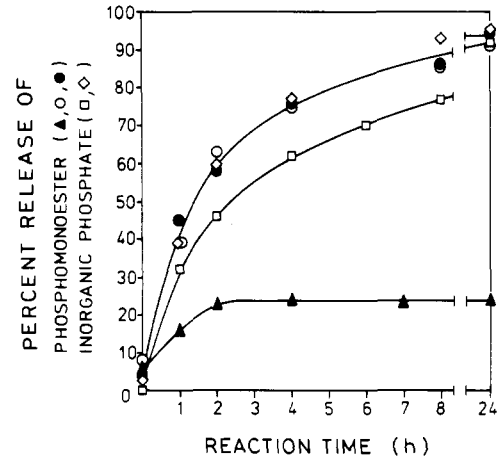

FIGURE 5: Time course of β elimination. Periodate-oxidized K2 antigen (▲), LTA [(○) IV and (◇) V of Figure 2], UMP (●), and native glyceraldehyde 3-phosphate (□) were incubated in 0.1 M triethylammonium carbonate buffer, pH 10.5, at 25 °C. The reaction was followed by measuring the released phosphomonoester or inorganic phosphate. One hundred percent refers to the total phosphorus of the samples.

For analysis, polyol phosphates were separated from free polyols by chromatography on DEAE-Sephacel. Gas–liquid chromatography of the trimethylsilylated products separated two minor peaks with the retention times of α- and β-glycerophosphates and a major peak with a retention time of 0.94 relative to that of D-erythritol 4-phosphate. Enzymic analysis revealed *sn*-glycero-3-phosphate which accounted for 45% of total glycerophosphate (Table I). Since the rest was β-glycerophosphate, the glycerophosphate of K2 antigen had the *sn*-glycero-3-phosphate stereochemical configuration.

Release of α-Glycerophosphate from Periodate-Oxidized K2 Antigen by β Elimination and Subsequent Hydrazinolysis. The structure-dependent low recovery in the above degradative sequence of α-glycerophosphate prompted us to try its release from the periodate-oxidized K2 antigen by elimination reactions. As outlined above, β elimination removes the phosphate from repeating unit IIa (Figure 1); subsequent hydrazinolysis could be expected to break the phosphodiester bond at repeating unit I (Brown et al., 1961; LeCocq & Ballou, 1964) and to remove the fragments of the oxidized galactosyl residues from glycerol (Brundish et al., 1965), resulting in the release of glycerophosphate. Only the phosphodiester bond at unit IIb, if this was present, seemed to be stable as erythrose 4-phosphate largely resisted both elimination reactions (data not shown).

β Elimination. K2 antigen and reference compounds were oxidized with $NaIO_4$ under the controlled conditions described above. After addition of ethylene glycol, the pH was immediately adjusted to 10.5. The time course of elimination at 25 °C is shown in Figure 5. In the case of K2 antigen, the reaction was complete in less than 3 h, with 24% of the total

Table II: Analyses of the Phosphorus-Containing Products Obtained from K2 Antigen and LTA by Periodate Oxidation, β Elimination, and Hydrazinolysis

parent compd	molar ratios to phosphorus: phosphomonoester[c]	glycerol[c]	α-glycerophosphate[d]	sn-glycero-3-phosphate[e]
E. coli K2 antigen	0.91[a] 0.96[b]	0.90[a] 0.93[b]	0.60[a] 0.87[b]	0.59[a] 0.88[b]
LTA, native	0.93[a] 0.99[b]	0.92[a] 0.96[b]	0.60[a] 0.94[b]	<0.01[a] <0.01[b]
LTA, degalactosylated[f]	0.95[a]	0.82[a]	0.82[a]	<0.01[a]

[a] Analysis after hydrazinolysis. [b] Analysis after final alkaline treatment (see text). [c] Measured after hydrolysis with acid phosphomonoesterase. Glycerol was determined by a glycerokinase-glycerol phosphate dehydrogenase assay. [d] Measured as inorganic phosphate after periodate oxidation and subsequent hydrazinolysis. [e] Measured with *sn*-glycero-3-phosphate dehydrogenase. [f] See Figure 2, IV.

Table III: Chromatographic Identification of the Phosphorus-Containing Products, Obtained from Periodate-Oxidized K2 Antigen by β Elimination and Subsequent Hydrazinolysis

	R_f[a]	t_R[b] (min)
reference compounds		
1(3),2-glycerophosphate	0.56	4.2
β-glycerophosphate	0.30	7.2
α-glycerophosphate	0.26	7.8
degradation products of K2 antigen	0.45,[c] 0.32,[c] 0.26	7.8

[a] Thin-layer chromatography on precoated cellulose plates developed with propan-1-ol–ammonia–water (6:3:1 v/v). [b] Gas–liquid chromatography of the trimethylsilylated products; t_R, retention time. [c] No longer detectable after alkaline treatment (see text).

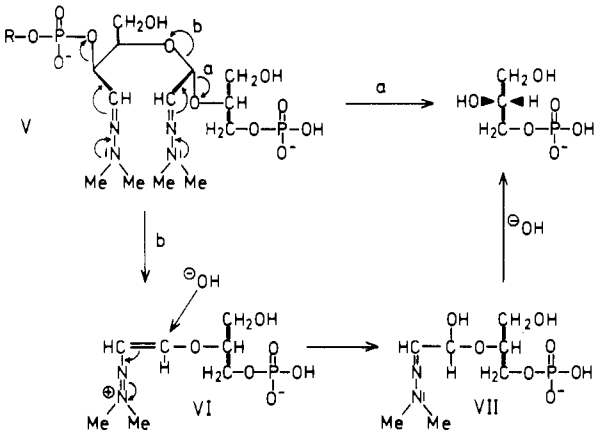

FIGURE 6: Proposed mechanism for the release of α-glycerophosphate from periodate-oxidized K2 antigen by hydrazinolysis and subsequent alkaline treatment.

phosphorus being converted into monoester. With LTA, as expected from its structure (Figure 2), more than 90% of the phosphodiester was opened. The reaction rate was considerably less than that with K2 antigen and similar to that of oxidized UMP. Control experiments with oxidized di-α,α′-glycerophosphate (data not shown) ensured that phosphodiester bonds as such and in the α position to carbonyl groups were stable. These results confirm that only the phosphodiester bond at repeating unit IIa of K2 antigen was opened. The observed higher reactivity suggests that the hydroxyl group at C-6 adjacent to the phosphodiester assisted the elimination reaction, presumably by hydrogen bonding.

Hydrazinolysis and Analysis of the Products. After β elimination, the products of K2 antigen and LTA were subjected to hydrazinolysis as described under Materials and Methods. The phosphorus-containing products were purified by column chromatography on DEAE-Sephacel from which they eluted as a single peak. They were analyzed as shown in Table II. In the case of K2 antigen, the phosphomonoester/phosphorus ratio of 0.91 indicated that most of the phosphodiester bonds which had resisted β elimination (Figure 5) were opened by hydrazinolysis. As judged from the approximately equimolar amounts of inorganic phosphate and glycerol, which were released by phosphomonoesterase, hydrazinolysis also seemed to have effectively removed the fragments of the oxidized galactosyl residues from glycerophosphate. In the case of K2 antigen and native LTA, however, only 60% of the glycerophosphate was susceptible to periodate oxidation. Nevertheless, the oxidizable fraction consisted exclusively of either *sn*-glycero-3-phosphate or *sn*-glycero-1-phosphate, which indicated that isomerization had not occurred. In accordance with this, neither gas–liquid nor thin-layer chromatography revealed the presence of β-glycerophosphate or cyclic 1(3),2-glycerophosphate (Table III). However, on thin-layer plates besides α-glycerophosphate, two fainter phosphate-positive spots of faster mobility were observed.

After alkaline treatment (0.1 M NaOH, 100 °C, 2 h), these two spots were no longer detectable, and as shown in Table II, most of the previously nonoxidizable glycerophosphate had been converted into either *sn*-glycero-3-phosphate or *sn*-glycero-1-phosphate.

In the case of LTA, a higher proportion of periodate-reactive glycerophosphate was released by hydrazinolysis (Table II) when before periodate oxidation the monogalactosyl residues, adjacent to the phosphodiester bonds, were removed (Figure 2, IV).

To explain these observations, we suggest, as shown in Figure 6, that the hydrazone at position 2 of V initiated two reactions: elimination of the acetal bond to give α-glycerophosphate and elimination of the ring oxygen to give the substituted glycerophosphate VI. To the latter, on alkali treatment, OH^- may be added and the resultant glyoxal hydrazone semiacetal (VII) hydrolyzed to yield α-glycerophosphate. Since on treatment with phosphomonoesterase glycerol was apparently released from VI (Table II, lane 2), the neighboring phosphate group may, at slightly acid pH, have interfered with the addition of OH^-. This would be in accordance with the higher yield of α-glycerophosphate after hydrazinolysis of degalactosylated LTA because in this case the nucleophilic addition occurred at a greater distance from the phosphate group (cf. Figure 2, IV).

Distribution of Galactofuranosyl and Galactopyranosyl Residues in K2 Antigen. The selective break on β elimination of the phosphodiester bond at position 5 of galactofuranosyl residues was used to study the arrangement of the pyranosidic and furanosidic galactosyl residues in K2 antigen.

After oxidation and treatment at pH 10.5, the pH was adjusted to 7 and the mixture chromatographed on a column of Sephadex G-50. For comparison, in a separate run native K2 antigen was chromatographed on the same column. As shown in Figure 7, the native polymer appeared as a single peak near the void volume of the column, whereas the β elimination products gave three incompletely separated peaks (I–III) which accounted for 24%, 58%, and 18% of the

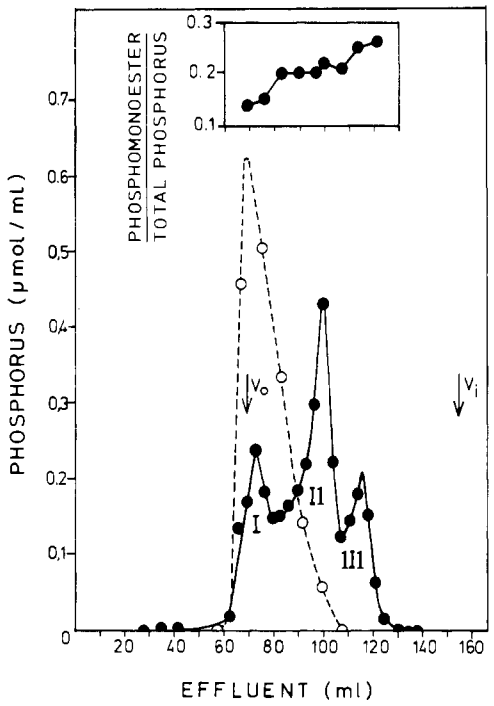

FIGURE 7: Gel permeation chromatography of native K2 antigen (○) and the β-elimination fragments of the periodate-oxidized polymer (●). Native and degraded K2 antigens (phosphomonoester/phosphorus 0.21) were applied to a column of Sephadex G-50 (0.25 × 100 cm) and eluted at 4 °C with water at 10 mL/h. Fractions of 3.5 mL were collected and analyzed for phosphorus. Every two fractions of the degraded polymer were combined and analyzed for phosphomonoester and total phosphorus, the ratios of which are shown in the inset.

phosphorus of the starting material.

Every two fractions of the fragmented K2 antigen were combined and analyzed for phosphomonoester and phosphate, the ratios of which are given in the inset to Figure 7. They were 0.14 and 0.15 in peak I, 0.20–0.22 in peak II, and 0.25 and 0.26 in peak III. These results clearly indicated that galactofuranosyl residues were not present in separate chains, for in this case a single fragment with a phosphomonoester/phosphate ratio of 1 would be expected near the inner volume of the column. Provided that all β elimination fragments terminated in a phosphomonoester, the phosphomonoester/phosphate ratios suggest that furanosidic and pyranosidic glycosyl residues were arranged in the sequences Gal*f*(*p*)GroP–(Gal*p*GroP)$_n$–Gal*f*GroP–, –Gal*f*GroP–(Gal*p*GroP)$_n$–Gal*f*GroP–, and/or –Gal*f*GroP–(Gal*p*GroP)$_n$–Gal*f*X, where *n* is 6, 4, and 3 for the fragments of peaks I, II, and III, respectively, and X is a phosphate-free residue or sequence at the "reducing" end of the native chain.

Discussion

In the present study we have shown that the glycerophosphates of *E. coli* K2 capsular antigen have the *sn*-glycero-3-phosphate configuration. For liberation of the glycerophosphate from the chain, the described degradative sequence comprising elimination reactions proved superior to the previously used combination of Smith degradation and alkaline phosphodiester cleavage. Even in the case of the structurally more favorable LTA, only 20% of the chain glycerophosphate was released as α-glycerophosphate with the latter method (Koch & Fischer, 1978) as compared to 90% with the new one.

The elimination reactions also provided information as to the chain structure of K2 antigen. The cleavage of 90% of the phosphodiester bonds by β elimination and hydrazinolysis is consistent with an unbranched structure which was previously suggested from the analysis of the alkali hydrolysis products (Jann et al., 1980). β elimination allowed location of the phosphodiester bond at C-5 of the galactofuranosyl residues, and the observed fragmentation pattern showed that galactofuranosyl residues alternate with galactopyranosyl residues in the same chain. Chain-length determinations of these fragments suggested the sequences Gal*f*(*p*)GroP–(Gal*p*GroP)$_n$–Gal*f*GroP–, –Gal*f*GroP–(Gal*p*GroP)$_n$–Gal*f*GroP–, and/or –Gal*f*GroP–(Gal*p*GroP)$_n$–Gal*f*X, where *n* is 6, 4, and 3, respectively. The distribution of the furanosidic residues can be more or less regular, depending on whether a single or all values of *n* belong to each sequence.

Containing *sn*-glycero-3-phosphate residues, K2 antigen is reminiscent of teichoic acid rather than lipoteichoic acid, which are made up by *sn*-glycero-3-phosphate and *sn*-glycero-1-phosphate residues, respectively (Archibald & Coapes, 1971; Koch & Fischer, 1978; Fischer, 1981). By biosynthetic studies of various compounds, it has been established that *sn*-glycero-3-phosphate is derived from CDP-glycerol (Burger & Glaser, 1964, 1966; Coley et al., 1978), whereas *sn*-glycero-1-phosphate is transferred from phosphatidylglycerol (Veerkamp, 1976; Emdur & Chiu, 1975; Kennedy et al., 1976; Ganfield & Pieringer, 1980). We may therefore suggest that CDP-glycerol, not yet looked for in Gram-negative bacteria, serves as the glycerophosphate donor in the biosynthesis of K2 antigen. UDP-galactofuranose (Trejo et al., 1970, 1971), a rare sugar nucleotide in nature, will also be required.

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Transient Kinetics of Adenosine 5′-Diphosphate and Adenosine 5′-(β,γ-Imidotriphosphate) Binding to Subfragment 1 and Actosubfragment 1†

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ABSTRACT: The kinetics of binding of the nonhydrolyzable nucleotides adenosine 5′-diphosphate (ADP) and adenosine 5′-(β,γ-imidotriphosphate) (AMP-PNP) to myosin subfragment 1 (SF-1) and actosubfragment 1 (acto-SF-1) were reinvestigated. The binding of these ligands to SF-1 can be described by

$$M_0 + L \overset{K_0}{\rightleftharpoons} M_0{\cdot}L \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} M_1{\cdot}L \overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}} M_{12}{\cdot}L$$

The nucleotide binds in a rapid equilibrium step (K_0), followed by two first-order fluorescence transitions with $k_1 + k_{-1} \gg k_2 + k_{-2}$. The rates and amplitudes of the fluorescence transitions are different for ADP and AMP-PNP and in turn can be distinguished from the corresponding steps involved in adenosine 5′-triphosphate (ATP) binding. The similarity in the maximum rate of the observed fluorescence signal for ADP and ATP binding to SF-1 in 0.1 M KCl is fortuitous as the maximum rates differ greatly at higher ionic strength. Under favorable conditions of high ionic strength where the amplitude of the fluorescence enhancement is large, the binding of AMP-PNP to acto-SF-1 gave a fluorescence change prior to dissociation, followed by a second fluorescence transition at the same rate as the dissociation of the proteins. Thus a conformation change precedes the nucleotide-induced dissociation of actomyosin. At least three acto-SF-1–nucleotide complexes are necessary to explain the kinetic behavior.

The reactions of nonhydrolyzable nucleotides with myosin and actomyosin have been extensively investigated particularly in terms of partial reactions, which yield information on the steps of the hydrolysis cycle. The nucleotides ADP[1] and AMP-PNP as well as pyrophosphate dissociate actomyosin, and the mechanism is expected to resemble the steps in which ATP produces dissociation prior to hydrolysis. The binding of ADP or AMP-PNP to SF-1 gives a tryptophan fluorescence signal of smaller amplitude (Bagshaw & Trentham, 1974; Bagshaw et al., 1974) and a proton burst of the same amplitude as for ATP (Bagshaw & Trentham, 1974; Koretz & Taylor, 1975; Chock, 1979; Marsh et al., 1977).

Information obtained from the binding of ADP and AMP-PNP to SF-1 was used as evidence for the assignment of rate constants in the ATPase reaction by Bagshaw & Trentham (1974):

$$M + ATP \overset{1}{\rightleftharpoons} M{\cdot}ATP \overset{2}{\rightleftharpoons} M{\cdot}ATP^* \overset{3}{\rightleftharpoons} M{\cdot}Pr^{**} \overset{4}{\rightleftharpoons} M{\cdot}ADP^* \overset{5}{\rightleftharpoons} M{\cdot}ADP \overset{6}{\rightleftharpoons} M + ADP \quad (1)$$

Asterisks refer to states of enhanced protein fluorescence, and M refers to a myosin head, or subfragment 1 (SF-1). Step 4 is rate limiting at 20 °C, and steps 5 and 6 are the reversal of ADP binding. Since the maximum rates of the fluorescence enhancement for ATP and ADP binding to SF-1 are equal in 0.1 M KCl, Bagshaw and Trentham suggested that this rate was a measure of k_2 or k_{-5} as these two steps have in common a transition of a bound nucleotide. The rate of the hydrolysis step (k_3) was assumed to be much greater than k_2, since the observed fluorescence transient fitted a single exponential reasonably well. The amplitude of the fluorescence change obtained when AMP-PNP binds to SF-1 was half of the change obtained with ATP. This suggested that nonhydrolyzable nucleotides bind to SF-1 in two steps, while there is a third step in the reaction with ATP in which hydrolysis occurs accompanied by an additional fluorescence increase. Measurements of fast proton release (Koretz & Taylor, 1975; Chock, 1979) also lead to similar values for the maximum rate constant and for the amount of protons released by ADP and ATP. The proton data provided additional support for the assignment of rate constants in the Bagshaw–Trentham scheme and for the release of a fraction of a proton in steps 2 and 5. This scheme provided a satisfactory synthesis of the available evidence, but recent studies have shown that a reassignment of the rate constants in the ATP binding scheme

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[1] Abbreviations: ADP, adenosine 5′-diphosphate; AMP-PNP, adenosine 5′-(β,γ-imidotriphosphate); ATP, adenosine 5′-triphosphate; SF-1, myosin subfragment 1; ATPase, adenosinetriphosphatase; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; Mes, 4-morpholineethanesulfonic acid; acto-SF-1, actosubfragment 1; DEAE, diethylaminoethyl.

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