A Single Point Mutation in 3-Deoxy-d-manno-octulosonate-8-phosphate Synthase Is Responsible for Temperature Sensitivity in a Mutant Strain of Salmonella typhimurium

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Salmonella typhimurium mutants conditionally deficient in 3-deoxy-d-manno-octulosonate-8-phosphate (KDO8P) synthase activity play a central role in our understanding of lipopolysaccharide function in enteric bacteria. The detailed characterization of KDO8P synthase from such a mutant, however, has not been previously reported. To address this issue KDO8P synthase from S. typhimurium AG701 and from a related temperature-sensitive strain (S. typhimurium AG701i50) have been overexpressed in Escherichia coli and purified to homogeneity. The enzyme from the temperature-sensitive strain has a single proline to serine substitution at position 145, leading to an increase in $K_m$ for both substrates, d-arabinose 5-phosphate and phosphoenolpyruvate. Analytical gel filtration and native polyacrylamide gel electrophoresis indicate that this enzyme also has an altered oligomeric state. These observations are rationalized through an examination of the structure of E. coli KDO8P synthase, which has 95% sequence identity to the enzyme from S. typhimurium.

The eight carbon acidic sugar 3-deoxy-d-manno-octulosonate (KDO) is an integral component of the lipopolysaccharide (LPS) of Gram-negative bacteria (1). This unusual sugar is the first component of the oligosaccharide core region that links lipid A to the O-antigen (2) (Fig. 1). KDO is incorporated into the LPS in four steps from d-arabinose 5-phosphate (A5P) and phosphoenolpyruvate (PEP). The first step is the condensation of A5P and PEP to form KDO 8-phosphate (KDO8P) (3). The resulting phosphorylated monosaccharide is first converted to free KDO and then activated with cytosine triphosphate to form CMP-KDO (4). A single step of KDO synthesis leads to bacteria whose normal growth and function have been compromised (8, 9). This finding, coupled with the localization of KDO to only Gram-negative organisms and plants (10), suggests that KDO8P synthase is a valuable therapeutic target for the development of novel antibacterial agents.

KDO8P synthase from E. coli has been well characterized over the past several years, and although the mechanism of action has not been fully elucidated, several key aspects of the enzymatic reaction are known. KDO8P synthase catalyzes the addition of the si face of PEP onto the re face of A5P, with the concomitant production of inorganic phosphate (11, 12). This reaction is unusual in that phosphate is released via C–O bond cleavage, rather than via the more common P–O bond cleavage (13, 14). KDO8P synthase is specific for A5P as the phosphorylated monosaccharide substrate and displays no requirement for a metal cofactor. Two mechanisms have been proposed for the condensation reaction as follows: (a) direct attack by water at C-2 of PEP to form a linear intermediate and (b) internal attack by a hydroxyl to form a cyclic intermediate (Fig. 2). Results from inhibition studies with both cyclic and acyclic product analogues (15, 16) have failed to differentiate between these mechanisms, and although no direct evidence exists, a recent structure determination favors the linear intermediate (17). In contrast to the numerous phenotypic studies on temperature-sensitive S. typhimurium mutants, and despite the prominent role of these experiments in rationalizing the study of KDO8P synthase and the wealth of knowledge concerning Escherichia coli KDO8P synthase, the nature of the S. typhimurium KDO8P synthase mutation at the enzymatic level has never been investigated. S. typhimurium AG701i50 is unable to grow at 42°C and has been used in complementation experiments to identify KDO8P synthase activity encoded in DNA libraries (18–20). This suggests that strain AG701i50 encodes for an altered KDO8P synthase, but this has never been proven experimentally. The parent strain of AG701i50 (S. typhi-
KDO8P Synthase from Temperature-sensitive S. typhimurium

The presence of the desired plasmid in selected transformants was verified by restriction mapping.

Growth Characteristics—The following procedures were performed in media without antibiotic supplementation. A growing culture of S. typhimurium in LB medium \( \Delta \text{amp} \) was diluted 25-fold into 2X YT medium that was equilibrated at 37 °C. Growth in the culture was grown with vigorous shaking and the \( \Delta \text{amp} \) monitored as a function of time. In experiments with exogenous A5P, the growth medium was supplemented with the appropriate concentration of monosaccharide immediately prior to inoculation.

**Purification of KDO8P Synthase—** E. coli BL21(DE3) cells containing the plasmid (described above) were grown at 37 °C with vigorous shaking. When this culture reached an A\text{0} of 0.6–0.8, isopropyl-\( \beta \)-thiogalactoside was added to a final concentration of 0.4 mM. Cells were harvested by centrifugation 4 h following induction. The following steps were performed at 4 °C. Pelleted cells were suspended (1 g per 5 ml) in 20 mM Tris-Cl, 0.2 mM DTT, pH 7.6 (Buffer A), and sonicated for 90 s (30-s pulses with a 90-s rest between pulses). The crude extract was centrifuged to remove cellular debris, and the supernatant was saved. The resultant pellet was suspended, sonicated, and centrifuged as above. The supernatants were combined and loaded onto a 5-ml Econo-Pac High Q cartridge (Bio-Rad) at 1.0 ml/min. The column was washed with 30 ml of Buffer A, and then a linear gradient of 0–500 mM KCl in Buffer A was over applied, and finally 0.6–1.2 ml of Buffer B, pH 6.5, by ultrafiltration. The enzyme was then aliquoted and stored at –80 °C. KDO8P synthase obtained in this manner is ~95% pure by SDS-polyacrylamide gel electrophoresis analysis.

Characterization of the KDO8P Synthase Reaction Product—** Purified wild-type KDO8P synthase (115 \( \mu \)g, 3.8 nmol) was mixed with 11 mM A5P (22 \( \mu \)mol), 13 mM PEP (26 \( \mu \)mol), and 100 mM Tris acetate, pH 7.4, in a total volume of 2.0 ml. The mixture was incubated at 37 °C for 2 h and then quenched with 10% trichloroacetic acid (500 \( \mu \)l). Purification of the resultant phosphorylated monosaccharide was as previously reported (23). The \( ^{1}H \) NMR spectrum of the product was recorded on a Bruker Avance DRX 500 at 500 MHz using the Watergate gradient suppression program and found to be identical to that previously reported for KDO8P (23).

Temperature Dependence of Purified KDO8P Synthase Activity—** PEP, A5P, and Tris acetate, pH 7.4 (45 \( \mu \)l), were incubated at the indicated temperature (15, 20, 25, 30, 35, 40, or 45 °C) for 3 min. The reaction was initiated by the addition of a 5-\( \mu \)l aliquot of 1 mM KDO8P synthase prepared in 25 mM sodium succinate, 5 mM EDTA, 5 mM DTT, pH 6.5 (held at 4 °C), to give final concentrations of 1 or 5 mM PEP, 1 or 5 mM A5P, 100 mM Tris acetate, and 5 mM enzyme. The reaction was quenched after a 1–15-min incubation by the addition of 10% trichloroacetic acid (50 \( \mu \)l). A 90-\( \mu \)l aliquot of the quenched reaction mixture was subjected to the periodate-thiobarbituric acid assay, and the amount of product released was determined using \( e = 103,000 \) 1 cm\(^{-1}\) at 549 nm (23). The reaction time was varied to maintain the extent of reaction at <10%.

Thermal Stability of KDO8P Synthase—** A 1 \( \mu \)l solution of wild-type or P145S enzyme was prepared in 25 mM sodium succinate, 5 mM EDTA, 5 mM DTT, pH 6.5, at 4 °C and assayed as above (5 mM PEP, 5 mM A5P, 37 °C). The enzyme solution was moved to 37 °C, and 5-\( \mu \)l aliquots were removed and assayed every 15 min for 1 h. The enzyme solution was then returned to 4 °C and incubated for 1 h and finally assayed again as above.

Determination of Steady State Kinetic Parameters from Progress Curves of PEP Disappearance—Spectra were recorded either with a Hewlett-Packard 8435 or with a Cary 3 Bio UV-Visible Spectrophotometer, each with a jacketed cuvette holder. Extent of reaction was determined from a non-linear regression of the reaction progress curve (24) using KaleidaGraph 3.08d (Synergy Software). \( k_{\text{cat}} \) was determined from \( k_{\text{cat}} = V_{\text{max}}/E_{0} \). All reactions were performed in 100 mM Tris acetate, pH 7.4, at either 25 or 37 °C. A typical reaction buffer, 0.6–1.2 \( \mu \)l of wild-type or KDO8P synthase with 5 mM A5P were incubated at 37 °C for 5 min. The reaction was initiated by the addition of a 20-\( \mu \)l aliquot of A5P for a final volume of 1.0 ml. The reaction was vortexed and transferred to a temperature-equilibrated cuvette and data were recorded for 3–5 min in 5–15-s intervals. For an experiment measuring the kinetic parameters for A5P, the initial concentration of PEP was 20 \( \times \) \( K_{m} \), the initial concentration of A5P was 3 \( \times \) \( K_{m} \) and the

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**EXPERIMENTAL PROCEDURES**

**Materials—** Phosphoenolpyruvate mono(cyclohexyl ammonium) salt, thioarbituric acid, and Tris base were from Sigma. \( \alpha \)-Aminobutyric acid, 5-phosphate was prepared as described by Volk (21) and purified by anion exchange chromatography to yield the monolithium salt. Cell media reagents were from Difco. All media contained 100 \( \mu \)g/ml ampicillin unless otherwise indicated. All other chemicals were from Fisher. Restriction enzymes were from New England Biolabs. Centriprep-10 centrifugal concentrators were from Millipore. Primer synthesis and DNA sequencing was performed by the University of Michigan Biomedical Research Core Facilities. S. typhimurium AG701 and AG701i50 were a generous gift from Dr. Werner Brabetz, Forschungszentrum Borstel, Borstel, Germany. XLI-Blue (Stratagene) and BL21(DE3) (Novagen) E. coli strains were purchased as chemically competent preparations.

Construction of Plasmids—** The following procedure was used with both S. typhimurium AG701 and S. typhimurium AG701i50. Standard polymerase chain reaction methodologies were used to amplify the putative kdsA gene from S. typhimurium genomic DNA. The forward primer was 5\'-AGTCTAGATCTTTGAAAACAAAAAGTGGTTAC- ATTGGCG-3\' and the reverse primer was 5\'-GATTCTGAAATTCCAGGCATTG-GGCTG-GC-3\' (gene sequences underlined). The amplification product was isolated, restricted with NdeI and BamHI (italic), and ligated into similarly treated pT7-7 (22). The ligation mixture was used to transform E. coli BL1-Blue cells. Plasmid DNA was isolated from several positive transformants and screened by restriction mapping. Those plasmids with the expected restriction pattern were subjected to DNA sequencing to confirm the presence of the desired gene. The plasmid arising from AG701 genomic DNA was designated pT7-7-kdsA-701, whereas that from AG701i50 was designated pT7-7-kdsA-701i50. These plasmids were then used to transform E. coli BL21(DE3) cells. Finally, the presence of the desired plasmid was verified by additional restriction mapping.

Electroporation—**Electroporation was achieved in a Bio-Rad E. coli Pulser. S. typhimurium AG701i50 cells were prepared and transformed with the desired plasmid according to the manufacturer’s instructions.

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**FIG. 1. Overview of bacterial lipopolysaccharide structure.** Non-carbohydrate modifications are not shown. Dashed lines indicate conserved substituents. Hep, \( \alpha \)-glycerol-\( \beta \)-manno-heptose. Regions not shown to scale.
reaction was followed to 95% completion. The extent of reaction of PEP was <5%. The relative concentrations of PEP and A5P were reversed to measure the parameters for PEP. No product inhibition was observed over the course of the reaction.

Determination of Steady State Kinetic Parameters from Initial Rates of Product Formation—\( V_{\text{max}} \) and \( K_m \) values were determined from a nonlinear regression of (substrate concentration, initial velocity) data pairs fit to the Michaelis-Menten equation using KaleidaGraph 3.08d (Synergy Software). All reactions were performed in 100 mM Tris acetate, pH 7.4, at either 25 or 37 °C. Product formation was measured using either a discontinuous assay to monitor the appearance of KDO8P or a continuous coupled assay to monitor the appearance of inorganic phosphate (23). In the discontinuous assay, buffer, PEP, and A5P were incubated for 3 min at the desired temperature. The reaction was initiated by the addition of a 5-\( \mu \)l aliquot of enzyme (150–450 ng of P145S KDO8P synthase) for a final volume of 50 \( \mu \)l. After 3 min the reaction was quenched by the addition of 10% trichloroacetic acid (50 \( \mu \)l) and assayed using the periodate-thiobarbituric acid assay described earlier. In the continuous assay, buffer, PEP, 7-methylinosine (250 \( \mu \)M), P145S KDO8P synthase (180–360 ng), and purine nucleoside phosphorylase (10 \( \mu \)g) were incubated for 3 min at the desired temperature. The reaction was initiated by the addition of a 10-\( \mu \)l aliquot of A5P for a final volume of 200 \( \mu \)l. The reaction was vortexed, transferred to a temperature equilibrated cuvette, and data recorded at 280 nm for 2 min in 1-s intervals. The initial rate was determined from a linear fit to the change in absorbance over this time. For an experiment measuring the kinetic parameters for A5P, the initial concentration of PEP was 20\( \times \) \( K_m \) and the initial concentration of A5P ranged from 0.25\( \times \) \( K_m \) to 6\( \times \) \( K_m \). The extent of reaction of A5P was <10% at all substrate concentrations. The relative concentrations of PEP and A5P were reversed to measure the parameters for PEP.

Determination of Oligomeric State—Size exclusion chromatography was performed at ambient temperature on a Superose 12 (10/30) column (Amersham Pharmacia Biotech). The column was equilibrated at 0.5 ml/min with 50 mM Tris-HCl, 150 mM NaCl, pH 7.4; 100-800 mM NaCl; and then quantitated using a Personal Densitometer (Molecular Dynamics).

**RESULTS**

**Growth Characteristics and Complementation**—Growth curves for *S. typhimurium* AG701 and AG701i50 were generated at temperatures of 30 and 37 °C to verify their temperature sensitivity. The doubling time for AG701i50 is only slightly increased at 30 °C as compared with AG701 (Fig. 3A), but AG701i50 growth is severely retarded at 37 °C (Fig. 3B). The addition of exogenous A5P does not rescue the growth of AG701i50 at either temperature. AG701i50 growth can be partially restored by complementation with pT7-7-kdsa-701, which encodes KDO8P synthase from strain AG701. Transformation with pT7-7 confers antibiotic resistance to AG701i50 but has no effect on its growth characteristics.

Cloning, Sequencing, and Protein Purification—When this project was initiated little genomic sequence data for a strain of *S. typhimurium* was available. There did exist, however, a series of contigs for *Salmonella typhi* CT18, generated by the

**Fig. 2. Putative mechanisms for KDO8P formation.** A furanose intermediate is equally viable for Path b.
level in the regions arising from the cloning primers, the proteins expressed with this system are authentic KDO8P synthase from *S. typhimurium* AG701 and AG701i50.2 The sequence data for kdsA from *S. typhimurium* AG701 and AG701i50 reveals a single nucleotide difference between the gene from the two strains, resulting in a proline (CCG) to serine (TCG) point mutation at position 145. An amino acid sequence comparison reveals that this proline is conserved among KDO8P synthases from a number of organisms, suggesting that it is important for correct expression of enzymatic activity. In the following discussion KDO8P synthase from strain AG701 is referred to as wild-type enzyme and that from strain AG701i50 is referred to as P145S mutant enzyme. Both wild-type and P145S KDO8P synthase from *S. typhimurium* were successfully purified according to a procedure similar to that previously reported for KDO8P synthase from *E. coli* (14).

The wild-type enzyme has no metal ion requirement and synthesizes KDO8P from A5P and PEP (verified by 1H NMR).

**Effects of Temperature on Wild-type and P145S KDO8P Synthase**—The specific activity of both wild-type and P145S enzyme was determined at several temperatures (Fig. 4). In the presence of 1 mM A5P and 1 mM PEP the wild-type enzyme displays an exponential dependence on temperature, with an Arrhenius factor of 23.0 ± 0.5 kcal/mol. The P145S enzyme has reduced activity (~35% of wild-type) and a nearly identical Arrhenius factor (23 ± 2 kcal/mol) between 15 and 30 °C. Above 30 °C the activity of this enzyme decreases dramatically. Increasing the level of each substrate to 5 mM has no effect on the wild-type enzyme, but the activity of the P145S enzyme now parallels that of the wild-type up to 35 °C, where it remains constant.

The thermal stability of both wild-type and P145S KDO8P synthase was examined at 37 °C. In the absence of substrates, the wild-type enzyme retains full activity over the course of a 1-h incubation. The specific activity of the P145S enzyme is reduced by approximately 50% under identical conditions. Shifting the heated P145S enzyme to 4 °C for 1 h does not restore enzymatic activity. In the presence of 500 μM PEP, however, the P145S enzyme retains full activity when incubated at 37 °C. The inclusion of 500 μM A5P with the P145S enzyme does not have a stabilizing effect. The wild-type enzyme is not effected by the addition of either A5P or PEP.

The steady state kinetic parameters for both wild-type and P145S KDO8P synthase at 25 and 37 °C are reported in Table I. The values for the wild-type enzyme at 37 °C are similar to those for the *E. coli* enzyme (26). The *Km* values for both substrates are elevated in the P145S enzyme as compared with the wild-type enzyme, with a 3-fold increase in *Km* for A5P and a 90-fold increase in *Km* for PEP at 25 °C. The differences in *Km* are even larger at 37 °C, with increases of 30- and 390-fold for A5P and PEP, respectively. The *kcat* values for the P145S enzyme are approximately 30–40% that of the wild-type enzyme at both temperatures.

**Oligomerization State of Wild-type and P145S KDO8P Synthase**—The apparent molecular masses of both wild-type and mutant enzymes were determined by size exclusion chromatography. The wild-type enzyme migrates with an apparent molecular mass of 97 ± 2 kDa, for an oligomeric state of 3.1 monomers per oligomer. The P145S enzyme migrates as two poorly resolved peaks, having apparent molecular masses of 91 ± 2 and 53 ± 1 kDa. These masses indicate a structure of 2.9 monomers per oligomer and 1.7 monomers per oligomer, respectively.

This phenomenon was further investigated by determining the electrophoretic mobility of both wild-type and mutant enzymes under non-denaturing conditions (native polyacrylamide gel electrophoresis) at 15, 25, and 35 °C. The wild-type enzyme migrates as a single, well resolved band at each temperature. The P145S enzyme migrates as two species at both 15 and 25 °C, a fairly well resolved high molecular weight band and an asymmetric low molecular weight band that tails toward the low molecular weight side, with approximately 30% of the enzyme migrating as the high molecular weight species. At 35 °C the high molecular weight fraction of the P145S enzyme is barely discernible, and the low molecular weight band exhibits considerable smearing on the low molecular weight side.

**DISCUSSION**

The temperature-sensitive nature of *S. typhimurium* AG701i50 was verified by recording its growth characteristics at two different temperatures (Fig. 3). In contrast to the char-

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**Table I**

**Kinetic parameters for KDO8P synthase**

Parameters for the wild-type enzyme were determined from progress curve analysis of PEP disappearance. Those for the P145S enzyme were determined from analysis of the initial rates of product formation. Errors represent the S.D. of three to eight experiments. The variable substrate in each experiment is shown in parentheses.

| Parameter      | Wild-type enzyme | P145S enzyme |
|----------------|------------------|--------------|
|                | 25 °C            | 37 °C        | 25 °C           | 37 °C           |
| *Km* (A5P)     | 4.4 ± 0.2 μM     | 9.3 ± 0.6 μM | 14 ± 1 μM      | 280 ± 20 μM    |
| *Km* (PEP)     | 2.8 ± 0.1 μM     | 4.2 ± 0.2 μM | 260 ± 20 μM    | 1650 ± 70 μM   |
| *kcat* (A5P)   | 1.75 ± 0.04 s⁻¹  | 5.6 ± 0.2 s⁻¹| 0.74 ± 0.01 s⁻¹| 1.63 ± 0.04 s⁻¹|
| *kcat* (PEP)   | 2.08 ± 0.07 s⁻¹  | 6.7 ± 0.5 s⁻¹| 0.72 ± 0.02 s⁻¹| 2.02 ± 0.06 s⁻¹|
acterization of the temperature-sensitive strain of *S. typhimurium* described by Rick and Osborn (6), we were unable to rescue strain AG701i50 using exogenous A5P. Growth can be partially restored, however, by complementation with a plasmid encoding for wild-type KDO8P synthase. Although the KDO8P synthase gene in this plasmid is downstream from a T7 promoter, low levels of expression from similar plasmids have been achieved by *E. coli* RNA polymerase (27). The high level of sequence homology between *E. coli* and *S. typhimurium* RNA polymerases (70–90% identity) suggests that expression can also be achieved in AG701i50.

In an attempt to correlate the temperature-sensitive growth reported above with a defect in KDO8P synthase, the specific activity of each enzyme was measured as a function of temperature (Fig. 4). There are three possibilities that may explain the decrease in activity of the P145S enzyme relative to the wild-type enzyme with increasing temperature as follows: a relative decrease in *k*<sub>cat</sub>, a relative increase in *K*<sub>m</sub>, or a decrease in the amount of active enzyme. The latter possibility was investigated by measuring the thermal stability of the P145S enzyme at 37 °C. Although this enzyme is less stable than wild-type in the absence of substrates, the addition of as little as 500 μM PEP fully protects P145S KDO8P synthase against thermally induced inactivation. As the activity data shown in Fig. 4 was measured in the presence of either 1 or 5 mM PEP, it is unlikely that the decreased activity for the P145S enzyme results from a change in the fraction of active enzyme.

To differentiate between the remaining possibilities, we measured *k*<sub>cat</sub> and *K*<sub>m</sub> at 25 and 37 °C (Table I). The *k*<sub>cat</sub> values for the wild-type enzyme are what one would expect based on the data in Fig. 4, both in terms of their magnitude and temperature dependence. The *k*<sub>cat</sub> values for the P145S enzyme are lower than those for wild-type at 25 °C and show only a slight relative decrease upon shifting to 37 °C. The *K*<sub>m</sub> values for both substrates with the P145S enzyme are elevated at 25 °C and increase dramatically relative to wild-type at 37 °C. These results are consistent with the ability of increasing levels of substrate to restore activity to the P145S enzyme at 35 °C (Fig. 4) and suggest that an elevated *K*<sub>m</sub> is largely responsible for the observed temperature dependence. Although an increase in *K*<sub>m</sub> for A5P is not unexpected based on the work reported by Rick and Osborn (6), we were surprised to find such a dramatic reduction in the binding of PEP. This may explain why A5P alone is not sufficient to rescue the growth of AG701i50.

To investigate whether Pro-145 plays a structural role in KDO8P synthase, we determined the oligomeric state of both wild-type and P145S enzyme. The wild-type enzyme behaves much like the *E. coli* enzyme; both migrate as an apparent trimer during size exclusion chromatography (28). The P145S enzyme migrates as two species, an apparent trimer and a lower molecular weight species intermediate between a dimer and a monomer. This result was confirmed using native polyacrylamide gel electrophoresis; at both 15 and 25 °C the wild-type enzyme appears as a single band, whereas the P145S enzyme resolves into two distinct species. The fraction of P145S enzyme that migrates as the high molecular weight species (30%) is remarkably similar to the ratio of P145S to wild-type specific activity at these temperatures (35%, Fig. 4) as well as to the ratio of *k*<sub>cat</sub> values (30–40%, Table I). This suggests that the majority of enzymatic activity is associated with the high molecular weight species, although we cannot exclude the possibility that the low molecular weight species has some residual activity.

This hypothesis is further supported by the following two observations: 1) in the absence of substrates, the P145S en-
zyme progressively loses activity upon incubation at 37 °C, and 2) when electrophoresed at 35 °C the majority of the P145S enzyme is found as the low molecular weight species. The correlation between loss of activity and change in oligomerization state suggests that full expression of enzymatic activity is only achieved in correctly formed oligomers. As no activity is regained upon shifting the P145S enzyme to 4 °C following incubation at 37 °C in the absence of substrates, we conclude that these two species are not in dynamic equilibrium but that conversion from the high molecular weight form to the low molecular weight form of the enzyme is an essentially irreversible process. Although the ratio of high molecular weight species to low molecular weight species observed here may not reflect the in vivo distribution of states, and may in fact be an artifact of overexpression and purification, it is clear that this problem occurs only for KDO8P synthase from the temperature-sensitive strain. Thus the possibility that incorrectly formed oligomers with reduced activity contribute to the restricted growth at elevated temperatures cannot be excluded.

During the course of this work a 2.4Å resolution crystallographic structure of E. coli KDO8P synthase became available (17). The high level of sequence identity between the S. typhimurium and E. coli enzymes (93%), as well as their similar kinetic behavior and gel filtration mobility, suggests that their three-dimensional structures will also be highly similar. This allows us to interpret the solution chemistry results reported above in terms of this new structural information. Although the size exclusion chromatography results suggest that KDO8P synthase is a trimer, the crystal structure shows it to be a tetramer and reveals the interactions that define the quaternary structure of the enzyme. Recent electrospray ionization mass spectrometry experiments also show the E. coli enzyme to be a tetramer in solution.3 The altered oligomeric state of the P145S mutant along with the conserved nature of Pro-145 in KDO8P synthase suggests that Pro-145 is required for correct assembly of the active form of KDO8P synthase. This hypothesis is borne out by the crystal structure, which shows that Pro-145 forms a type III turn between sheet S5 and helix H5. Tetramer assembly is determined, in part, by interactions between helix H5 from one subunit and loop L2 from an adjacent subunit (Fig. 5). Disruption of these contacts is likely to result in a decrease in the number of properly formed oligomers.

The altered K_m values for the P145S enzyme can also be rationalized through examination of the structure of KDO8P synthase. The crystal structure of the E. coli enzyme was solved with two bound sulfate ions: SO4-1 is thought to occupy the phosphate-binding site for PEP, whereas SO4-2 is thought to occupy the phosphate-binding site for ASP. The SO4-1-binding site is determined in part by Lys-138 (17), which forms a 2.8Å hydrogen bond with SO4-1 and is positioned by sheet S5 (Fig. 6). The highly elevated K_m for PEP with the P145S mutant suggests that the binding energy provided by Lys-138 is largely lost in the mutant enzyme. This may be caused by a disruption in the placement of sheet S5 due to the inability of the mutant enzyme to form the correct turn between sheet S5 and helix H5. The interactions described above between helix H5 and loop L2 also provide an explanation for the increased K_m for ASP. The binding of SO4-2 is determined by the side chain of Arg-63 and by the side chain and backbone amide of Ser-64 (Fig. 6), both of which are contributed to the active site by loop L2 (17). The loss of proper contacts between helix H5 and loop L2 may result in non-optimal positioning of loop L2, leading to reduced affinity for ASP.

The experiments described above represent the first detailed investigation of an altered KDO8P synthase from a temperature-dependent strain of S. typhimurium. A single point mutation of proline to serine leads to a decrease in substrate binding for both ASP and PEP, as well as to reduced thermal stability in the absence of substrates. Whether these changes act singly or in concert to achieve the observed phenotype is unknown. Although many attempts at drug design are focused on identifying compounds that bind in place of natural substrates, these results suggest that the disruption of correct oligomer formation may also be a viable therapeutic target. This work also points out the value of re-evaluating previous results in the light of currently available experimental techniques. Our interest in the history of KDO8P synthase led us to explore more fully the cause of reduced activity in S. typhimurium mutants, and to apply advances in cloning and protein overexpression to answer questions first raised more than 25 years ago.

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